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PRINCIPAL INVESTIGATOR: Ebba U. Kurz, Ph.D.
Susan P. Lees-Miller, Ph.D.

CONTRACTING ORGANIZATION: University of Calgary
Calgary, Alberta, Canada T2N 1N4

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6. AUTHOR(S)
Ebba U. Kurz, Ph.D.
Susan P. Lees-Miller, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Calgary
Calgary, Alberta, Canada T2N 1N4

E-Mail: kurz@ucalgary.ca

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13. ABSTRACT (Maximum 200 Words)

Ataxia-telangiectasia mutated (ATM) is a serine/threonine protein kinase that acts as a master switch controlling the cell cycle in response to ionizing radiation-induced DNA double-strand breaks (DSBs). Carriers of *ATM* mutations are at increased risk for breast cancer. Since many anti-tumor chemotherapeutics used in breast cancer treatment have the capacity to induce DNA DSBs, I have investigated the requirement for ATM in the cellular response to these agents. I have previously identified doxorubicin as an agent that stimulates ATM autophosphorylation and the ATM-dependent phosphorylation of p53. I have now expanded these studies to examine numerous downstream effectors of ATM and, in all cases, observe a dependence on ATM for phosphorylation. These phosphorylation events are attenuated by pretreatment of cells with N-acetyl cysteine, suggesting a role for hydroxyl radicals in these events. My studies have now been expanded and I have observed doxorubicin-induced phosphorylation of a subset of downstream effectors of ATM in two human breast cancer cell lines. Studies are now underway to identify proteins that interact with ATM following drug treatment. Characterization of a role for ATM in the cellular response to anti-tumor chemotherapeutics could have significant implications for the treatment of breast cancer patients harboring mutations in *ATM*.

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Annual Summary

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Principal Investigator: Ebba U. Kurz, Ph.D.
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Introduction

ATM is a nuclear protein kinase required for the arrest of the cell cycle at G_1/S, S and G_2/M in response to ionizing radiation (IR)-induced DNA damage (reviewed in (Kurz and Lees-Miller, 2004)). Inherited defects in ATM lead to the development of ataxia telangiectasia (A-T), a progressive neurodegenerative disorder characterized by profound sensitivity to IR, cancer predisposition, immunodeficiency, and a progressive loss of motor control due to cerebellar ataxia (reviewed in (Lavin and Shiloh, 1997)). Although A-T is relatively rare, studies suggest that 1% of the normal population is heterozygous for *ATM* mutations and that *ATM* heterozygosity could play a more significant role than BRCA1 and BRCA2 in breast cancer (Khanna, 2000).

Exposure to IR causes DNA double-strand breaks leading to the activation of ATM in the cell (Kurz and Lees-Miller, 2004). Interestingly, many of the anticancer drugs used in the treatment of breast cancer also have the capacity to induce DNA double-strand breaks (Fritsche et al., 1993; Vock et al., 1998), however little has been known about the role of ATM in response to damage induced by these drugs. The first aim of my three-year training grant from the US Army Breast Cancer Research Program has been to examine the effects of DNA damaging chemotherapeutics on the activation of ATM *in vivo*. The continued experimental efforts of the previous year towards this aim are summarized in this report.

Body (Detailed Research Accomplishments)

The requirement for the serine/threonine protein kinase ATM has been extensively studied in the cellular response to IR-induced DNA damage; comparatively little has been known about the role of ATM in response to DNA-damaging anti-tumor chemotherapeutics. In the first year of funding of this grant (as detailed in my Annual Summary submitted in September 2003), my experimental efforts focused on evaluating the role of ATM in the cellular response to a broad range of these agents that are used in the treatment of breast cancer. Using paired ATM-proficient and ATM-deficient cell lines, these studies led to the identification of doxorubicin, a topoisomerase II poison, as a DNA-damaging chemotherapeutic that activated an extensive ATM signaling cascade in the cell. In response to doxorubicin treatment, we observed ATM autophosphorylation on serine 1981, ATM-dependent nuclear accumulation of p53 and ATM-dependent phosphorylation of p53 on seven serine residues, accompanied by an increased binding of p53 to its cognate DNA binding site. Treatment of cells with doxorubicin also led to the ATM-dependent phosphorylation of histone H2AX on serine 139. Although primarily regarded as a topoisomerase II poison, we have obtained evidence that reactive oxygen species, specifically hydroxyl radicals, participate in the rapid doxorubicin-mediated activation of these ATM-dependent pathways. Given the success of the first year of experiments, an abstract describing these studies was submitted and accepted for presentation at the Eighth International Workshop on Ataxia Telangiectasia held on Fraser Island, Queensland, Australia, September 10-14, 2003. At this exclusive meeting, my work was very well received and I was awarded a Young Investigator Award Shortly thereafter, I presented my work at the 2003 Annual Research Meeting of the Alberta Cancer Board held in Banff, Alberta, November 12-14, 2003.

At this meeting, I received the top poster prize in the post-doctoral category for my presentation on doxorubicin-induced activation of ATM signaling.

At this time, I was invited by Dr. Susan Lees-Miller to co-author a review entitled "DNA-damage induced activation of ATM and ATM-dependent signaling pathways" which appeared in a special monograph issue of *DNA Repair* entitled *Bridge over Broken Ends: The Cellular Response to DNA Breaks in Health and Disease* edited by Dr. Yossi Shiloh, the discoverer of the ATM gene. The ATM field, along with the understanding and identification of ATM-dependent signaling pathways, has undergone an explosion in the past two years. Writing this review article was a very worthwhile endeavor as it strengthened my comprehension of my chosen field of study by requiring the synthesis of a broad, complex and rapidly changing research area into a clear and concise written review.

Most important in this past year was my continued progress on my research project as funded by the USAMRMC. At the time of submission, due to the field's limited understanding of ATM-dependent signaling pathways, I proposed to look at a restricted number of downstream effectors of ATM. As reported last year, I initially expanded the repertoire of downstream molecules I examined by expanding my analysis of p53 to examine seven phosphoserine residues. Although p53 is an important target of ATM, activation of ATM results in the phosphorylation of a diverse array of downstream targets that participate in multiple cellular processes. Analysis of ATM-dependent phosphorylation of one substrate cannot provide an accurate picture of the complexity of the cellular response. To gain a broader perspective on the requirement for ATM in the early cellular response to doxorubicin, ATM-proficient and ATM-deficient cells were treated with doxorubicin (1 μM) and incubated for 60 or 120 minutes prior to extract preparation and immunoblotting with phosphospecific antisera that are now commercially available. Exposure to doxorubicin induced the ATM-dependent phosphorylation of numerous effectors in the ATM-signaling pathway, including Nbs1 (S343), SMC1 (S957), Chk1 (S317 and S345), and Chk2 (S33/35 and T68). In a manner similar to p53, preincubation with the hydroxyl radical scavenger N-acetyl cysteine attenuated the doxorubicin-induced phosphorylation at all of these sites, further supporting a role for hydroxyl radicals in the doxorubicin-induced activation of ATM-dependent pathways. These data are reported in a manuscript that has received positive reviews from the *Journal of Biological Chemistry (attached)*.

Although funding for this grant was through the Breast Cancer Research Program, defining a role for ATM in the cellular response to DNA-damaging chemotherapeutics required the use of paired ATM-proficient and ATM-deficient cell lines. Using these, I was able to identify doxorubicin as an anti-tumor chemotherapeutic that activates ATM-dependent signaling within the cell. With the knowledge gained from the ATM-proficient and ATM-deficient lymphoblastoid cell lines, I have now been able to examine the effects of doxorubicin in the breast cancer cell lines, MCF7 and MCF10A. Initially, I determined the dose and time requirements for doxorubicin experiments with these breast cancer cell lines. Subsequently, I have determined that doxorubicin induces ATM autophosphorylation on serine 1981, as well as the phosphorylation of numerous downstream effectors (Nbs1 (S343), SMC1 (S957), p53 (S15), Chk2 (T68)) in these cell lines. Interestingly, phosphorylation of Chk2 at serines 33/35 was detectable in MCF10A cells, but not in MCF7 cells. Unlike lymphoblastoid cells, no doxorubicin-induced phosphorylation of Chk1 was observed, however, Chk1 protein levels are significantly lower in the breast cancer cells than in lymphoblastoid cells. The significance of this observation is unknown.

One of the tasks outlined in the approved Statement of Work was to evaluate changes in cell cycle distribution following drug treatment. With the assistance of the University of Calgary Flow Cytometry core facility, ATM-proficient and ATM-deficient cells were treated with

doxorubicin (1 μM), harvested at 6, 12 and 24 hours post-treatment and stained with propidium iodide for flow cytometric analysis. Using this technique, I have determined that doxorubicin treatment induces a robust intra-S phase cell cycle arrest in ATM-proficient cells, whereas this arrest is delayed in ATM-deficient cells. Experiments evaluating the effect of doxorubicin treatment on cell cycle distribution in breast cancer cells are currently underway.

In the reviews of my manuscript describing the doxorubicin-induced activation of ATM-signaling pathways, the reviewing editor at the *Journal of Biological Chemistry* requested that I evaluate the effect of N-acetyl cysteine pretreatment on doxorubicin-induced phosphorylation of histone H2AX at serine 139. In our laboratory, we have had significant technical difficulties with the reproducibility of histone extraction from cells and immunoblotting for histone H2AX using a phosphospecific antibody. In addition, visualization of H2AX phosphorylation by immunofluorescence is deemed the 'gold standard' technique within the field. Consequently, Dr. Pauline Douglas in our laboratory has established an immunofluorescence protocol for the visualization of phosphorylated histone H2AX in adherent cells. However, my experiments required the adaptation of this technique to suspension cells. Consequently, I have optimized a protocol for preparing cytospins of suspension cells for use in immunofluorescence. In a combined effort, Dr. Douglas and I have now acquired the data requested by the reviewers and a revised manuscript will be submitted shortly to the *Journal of Biological Chemistry.*

The expansion of the project to evaluate a broader panel of downstream effectors in ATM-signaling pathways, the work-up of additional techniques for publication of these important findings and the opportunity to co-author an invited review of the ATM field has delayed the initiation of experiments to identify proteins that interact with ATM following treatment with IR or ATM-activating chemotherapeutics in breast cancer cells. It is anticipated that these experiments will begin within the next two months. Similarly, experiments examining the effects of doxorubicin on the activation of ATM in breast cancer cells (as described in Aim 1, Task 2) will be concluded within this time.

In addition to conducting the research described herein, my training has been enriched through my interactions with other members of the Cancer Biology Research Group at the University of Calgary. As a member of this research group, I continue to attend and participate in a weekly journal club and a weekly Work In Progress seminar series. I also participate in regular meetings with the Radiation Oncology group at the Tom Baker Cancer Centre. The purpose of these meetings is to enhance the interaction between basic research scientists studying DNA damage response pathways important in the cellular response to IR and the radiation oncologists and translational researchers at the regional cancer center. As a senior member of Dr. Lees-Miller's laboratory, I also mentor summer, undergraduate and graduate students in the laboratory.

Key Research Accomplishments

- I determined that ATM is required for phosphorylation of Nbs1 (S343), SMC1 (S957), Chk1 (S317, S345), and Chk2 (S33/35, T68) in response to doxorubicin treatment
- I determined that phosphorylation at these sites can be partially attenuated by pretreatment of cells with the hydroxyl radical scavenger, N-acetyl cysteine.
- I established, using immunofluorescence, that the phosphorylation of histone H2AX in response to doxorubicin is ATM-dependent only in the early time (60 min) after treatment and that a redundant or complementary kinase is induced by 120 min after treatment. Also, the early ATM-dependent component can be partially attenuated by pretreatment with N-acetyl cysteine.

- I determined the effects of doxorubicin treatment on cell proliferation and cell cycle distribution in ATM-proficient and ATM-deficient cell lines.
- I established dose and time requirements for doxorubicin experiments in the breast cancer cell lines, MCF7 and MCF10A
- I determined that doxorubicin induces ATM autophosphorylation on serine 1981 in MCF7 and MCF10A breast cancer cells
- I determined that doxorubicin induces the phosphorylation of Nbs1 (S343), SMC1 (S957), p53 (S15), and Chk2 (T68) in the breast cancer cell lines, MCF7 and MCF10A

Reportable Outcomes

1. My studies on the ATM-dependent effects of doxorubicin treatment were presented at the International Ataxia Telangiectasia Workshop held on Fraser Island, Queensland, Australia, September 10-14, 2003. The abstract was entitled: **Kurz, E.U.** and Lees-Miller, S.P. Doxorubicin induces ATM autophosphorylation and ATM-dependent phosphorylation of human p53 and histone H2AX: evidence for a role for reactive oxygen species? For this work, I was awarded a Young Investigator Award.
2. My work contributed to a second presentation at the International Ataxia Telangiectasia Workshop held on Fraser Island, Queensland, Australia, September 10-14, 2003. The abstract was entitled: Lees-Miller, S.P., Goodarzi, A.A., **Kurz, E.U.**, Siponen, M.I. and Ye, R. Characterization of ATM and ATM-dependent DNA damage response pathways.
3. My work was presented at the Alberta Cancer Board 2003 Annual Research Meeting held in Banff, Alberta, Canada, November 12-14, 2003. The abstract was entitled: **Kurz, E.U.** and Lees-Miller, S.P. Doxorubicin induces ATM autophosphorylation and ATM-dependent phosphorylation of human p53 and histone H2AX: evidence for a role for reactive oxygen species? At this meeting, I was awarded the top poster prize in the post-doctoral category for this work.
4. My studies contributed to the work described in: Ye, R., Goodarzi, A.A., **Kurz, E.U.**, Saito, S., Higashimoto, Y., Lavin, M.F., Appella, E., Anderson, C.W. and Lees-Miller, S.P. (2004) The isoflavonoids genistein and quercetin activate different stress signaling pathways as shown by analysis of site-specific phosphorylation of ATM, p53 and histone H2AX. *DNA Repair (Amst)* 3:235-244.
5. I co-authored an invited review: **Kurz, E.U.** and Lees-Miller S.P. (2004) DNA-damage induced activation of ATM and ATM-dependent signaling pathways. *DNA Repair (Amst)* 3:889-900. This appeared in a special monograph issue of *DNA Repair* entitled *Bridge over Broken Ends: The Cellular Response to DNA Breaks in Health and Disease* edited by Dr. Yossi Shiloh, the discoverer of the ATM gene.
6. My studies are described in the manuscript: **Kurz, E.U.**, Douglas, P. and Lees-Miller, S.P. Doxorubicin activates ATM-dependent phosphorylation of multiple downstream targets in part through the generation of reactive oxygen species. This manuscript has received positive reviews and is under revision for the *Journal of Biological Chemistry*.

Conclusions

It has been reported that mutations in *ATM* could account for up to 5% of breast cancers, thus ATM could play a more significant role in breast cancer than BRCA1 and BRCA2. Characterization of a role for ATM in the cellular response to anti-tumor chemotherapeutics

could have significant implications leading to modified treatment protocols with fewer side effects for breast cancer patients who carry mutations in *ATM*.

The research conducted in the second year of my three-year training grant from the U.S. Army Breast Cancer Research Program expanded upon the findings of Year 1 and identified numerous effectors in the ATM signaling pathway, including Nbs1, SMC1, Chk1 and Chk2, that are phosphorylated in an ATM-dependent manner following doxorubicin treatment. My observations have now been carried over to the study of the cellular responses to doxorubicin treatment in two breast cancer cell lines and the role of ATM in these responses. These fundamental findings have now paved the way for the identification of proteins in that interact with ATM in breast cancer cells following IR or doxorubicin treatment. These studies may provide clues as to the preferential predisposition of *ATM* heterozygotes to breast cancer.

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Appendices

Appendix 1: Abstract (Kurz and Lees-Miller) from the International Ataxia Telangiectasia Workshop, 2003.

Appendix 2: Abstract (Lees-Miller, et al.) from the International Ataxia Telangiectasia Workshop, 2003.

Appendix 3: Abstract (Kurz and Lees-Miller) from the Alberta Cancer Board Annual Research Meeting, 2003.

Appendix 4: Ye, R., Goodarzi, A.A., **Kurz, E.U.**, et al (2004) The isoflavonoids genistein and quercetin activate different stress signaling pathways as shown by analysis of site-specific phosphorylation of ATM, p53 and histone H2AX. *DNA Repair (Amst)* 3:235-244.

Appendix 5: **Kurz, E.U.** and Lees-Miller S.P. (2004) DNA-damage induced activation of ATM and ATM-dependent signaling pathways. *DNA Repair (Amst)* 3:889-900.

Appendix 6: **Kurz** *et al* manuscript submitted to the *Journal of Biological Chemistry*.

Appendix 7: Reviewer comments for **Kurz** et al. manuscript submitted to the *Journal of Biological Chemistry*.

Appendix 1: Abstract from the International Ataxia Telangiectasia Workshop
Fraser Island, Queensland, Australia September 10-14, 2003.

Doxorubicin induces ATM autophosphorylation and ATM-dependent phosphorylation of human p53 and histone H2AX: evidence for a role for reactive oxygen species?

Ebba U. Kurz and Susan P. Lees-Miller

Department of Biochemistry and Molecular Biology and Cancer Biology Research Group
University of Calgary, 3330 Hospital Drive NW, Calgary, AB Canada T2N 4N1

Although A-T is a relatively rare disorder, studies suggest that 1% of the normal population is heterozygous for mutations in ATM and that *ATM* heterozygosity could account for up to 5% of all breast cancers. The requirement for ATM in co-ordinating the cellular response to DNA damage induced by ionizing radiation has been studied extensively. Many of the anti-tumour chemotherapeutics commonly used in the treatment of breast cancer (doxorubicin, cyclophosphamide, methotrexate and 5-fluorouracil) cause, either directly or indirectly, DNA double-strand breaks. For breast cancer patients heterozygous for mutations in *ATM*, we speculated that exposure to these drugs could lead to a more profound manifestation of side effects or the increased incidence of secondary, treatment-related malignancies. At present, few DNA-damaging chemotherapeutics have been evaluated for their ability to stimulate ATM-mediated cellular responses.

We have investigated the requirement for ATM in the cellular response to doxorubicin (Adriamycin™), a topoisomerase II-stabilizing drug. Using several human ATM-positive and ATM-negative cell lines, we have observed ATM-dependent nuclear accumulation of p53 and ATM-dependent phosphorylation of p53 on seven serine residues. This was accompanied by an increased binding of p53 to its cognate binding site, suggesting a transcriptional competency of p53 to activate downstream effectors. An early step in the cellular response to ionizing radiation is the ATM-dependent phosphorylation of histone H2AX at the sites of DNA damage. Similarly, treatment of cells with doxorubicin led to ATM-dependent phosphorylation of histone H2AX on serine 139, suggestive of the induction of DNA double-strand breaks. Treatment of cells with doxorubicin also stimulated ATM autophosphorylation on serine 1981.

Although generally classified as a topoisomerase II-stabilizing drug that induces DNA double-strand breaks, doxorubicin can intercalate DNA and generate reactive oxygen species. Pre-treatment of cells with the superoxide scavenger, ascorbic acid, had no effect on the doxorubicin-induced phosphorylation and accumulation of p53. In contrast, pre-incubation of cells with the hydroxyl radical scavenger, N-acetyl cysteine, significantly attenuated the doxorubicin-mediated phosphorylation and accumulation of p53, suggesting that hydroxyl radicals may play an important role in doxorubicin-induced activation of ATM-dependent pathways.

Supported by the Alberta Cancer Board with funds from the Alberta Cancer Foundation, the United States Army (DAMD17-02-1-0318) and the National Cancer Institute of Canada (#11053) with funds from the Canadian Cancer Society.

Appendix 2: Abstract from the International Ataxia Telangiectasia Workshop
Fraser Island, Queensland, Australia September 10-14, 2003.

Characterization of ATM and ATM-dependent DNA damage response pathways

Susan P. Lees-Miller, Aaron A. Goodarzi, Ebba U. Kurz, Marina I. Siponen and Ruiqiong Ye

Department of Biochemistry and Molecular Biology, University of Calgary, Calgary, AB Canada T2N 4N1

Ataxia-telangiectasia mutated (ATM) is a 369 kDa polypeptide that is a member of the phosphatidyl inositol-3 kinase family of serine/threonine protein kinases (PIKKs). We previously reported the purification of ATM from human placenta (Chan et al, 2000). The purified protein had manganese dependent protein kinase activity and supported phosphorylated p53 on serine 15, however, its activity was not stimulated by DNA.

We have now purified microgram quantities of ATM from the nuclear pellet of HeLa cells. The purified HeLa cell ATM was highly active in vitro towards a variety of substrates including p53 and PHAS-I. Purified ATM kinase activity is stimulated by calf thymus DNA, single stranded closed circular M13 DNA, heparin sulphate, poly-lysine and poly-ADP ribose, suggesting that charged biological polymers stimulate ATM activity. Purified ATM from HeLa cells eluted as a monomer of 369 kDa on gel filtration and was phosphorylated on serine 1981. We have also characterized activation of ATM and DNA damage induced signaling pathways in response to a variety of DNA damaging agents including genistein, quercetin, etoposide and doxorubicin. While each of these DNA damaging agents induced phosphorylation of ATM on serine 1981, ATM was only required for phosphorylation of p53 in response to genistein and doxorubicin. We conclude that phosphorylation of ATM on serine 1981 is separate from ATM dependent phosphorylation of p53, and that different DNA damaging agents can activate distinct ATM-dependent and ATM-independent DNA damage signaling pathways.

Appendix 3: Abstract from the Alberta Cancer Board Annual Research Meeting 2003

Doxorubicin induces ATM autophosphorylation and ATM-dependent phosphorylation of human p53 and histone H2AX: evidence for a role for reactive oxygen species?

Ebba U. Kurz and Susan P. Lees-Miller
Department of Biochemistry and Molecular Biology and Cancer Biology Research Group, University of Calgary, Calgary, AB

Ataxia telangiectasia mutated (ATM) is a cellular protein kinase that acts as a master switch controlling if and when cell cycle progression arrests in response to ionizing radiation (IR). Exposure to IR results in an increase in the protein kinase activity of ATM, ATM-dependent up-regulation of p53 protein, and the direct and indirect phosphorylation of p53. In addition, ATM contributes to p53-independent mechanisms of cell cycle checkpoint activation. IR-induced DNA double-strand breaks (DSBs) are thought to be an important trigger leading to ATM activation. Since many anti-tumour chemotherapeutics also have the capacity to induce DNA DSBs, we have investigated the requirement for ATM in the cellular response to doxorubicin, a topoisomerase II-stabilizing drug. Using several human ATM-positive and ATM-negative cell lines, we have observed ATM-dependent nuclear accumulation of p53 and ATM-dependent phosphorylation of p53 on seven serine residues. This was accompanied by an increased binding of p53 to its cognate binding site. Treatment of cells with doxorubicin also stimulated ATM-dependent phosphorylation of histone H2AX on serine 139 and ATM autophosphorylation on serine 1981. Co-incubation of cells with N-acetyl cysteine attenuated the doxorubicin-induced effect, suggesting that hydroxyl radicals may play an important role in doxorubicin-induced activation of ATM-dependent pathways.

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The isoflavonoids genistein and quercetin activate different stress signaling pathways as shown by analysis of site-specific phosphorylation of ATM, p53 and histone H2AX

Ruiqiong Ye[a], Aaron A. Goodarzi[a], Ebba U. Kurz[a], Shin'ichi Saito[b], Yuichiro Higashimoto[b,1], Martin F. Lavin[c], Ettore Appella[b], Carl W. Anderson[d], Susan P. Lees-Miller[a,*]

[a] *Department of Biochemistry & Molecular Biology, University of Calgary, 3330 Hospital Drive, N.W., Calgary, Alta., Canada T2N 4N1*
[b] *Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA*
[c] *Queensland Institute of Medical Research, PO Royal Brisbane Hospital, Herston, Brisbane 4029, Qld., Australia*
[d] *Biology Department, Brookhaven National Laboratory, Upton, NY 11973, USA*

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Abstract

The ataxia-telangiectasia mutated (ATM) protein kinase is activated in response to ionizing radiation (IR) and activates downstream DNA-damage signaling pathways. Although the role of ATM in the cellular response to ionizing radiation has been well characterized, its role in response to other DNA-damaging agents is less well defined. We previously showed that genistein, a naturally occurring isoflavonoid, induced increased ATM protein kinase activity, ATM-dependent phosphorylation of p53 on serine 15 and activation of the DNA-binding properties of p53. Here, we show that genistein also induces phosphorylation of p53 at serines 6, 9, 20, 46, and 392, and that genistein-induced accumulation and phosphorylation of p53 is reduced in two ATM-deficient human cell lines. Also, we show that genistein induces phosphorylation of ATM on serine 1981 and phosphorylation of histone H2AX on serine 139. The related bioflavonoids, daidzein and biochanin A, did not induce either phosphorylation of p53 or ATM at these sites. Like genistein, quercetin induced phosphorylation of ATM on serine 1981, and ATM-dependent phosphorylation of histone H2AX on serine 139; however, p53 accumulation and phosphorylation on serines 6, 9, 15, 20, 46, and 392 occurred in ATM-deficient cells, indicating that ATM is not required for quercetin-induced phosphorylation of p53. Our data suggest that genistein and quercetin induce different DNA-damage induced signaling pathways that, in the case of genistein, are highly ATM-dependent but, in the case of quercetin, may be ATM-dependent only for some downstream targets.

Keywords: p53; Genistein; Quercetin; Isoflavonoid; DNA damage; Phosphorylation

1. Introduction

Ataxia-telangiectasia (A-T) is a rare inherited disease that is caused by dysfunction or loss of both copies of the ataxia-telangiectasia mutated (*ATM*) gene. The disease is characterized by progressive cerebellar degeneration, neuromuscular dysfunction, immunodeficiency, cancer predisposition, and genomic instability. Although the disease itself is relatively rare, individuals with one defective copy of *ATM* comprise 1–2% of the general population, and members of A-T families are at an elevated risk for developing cancer, in particular breast cancer (reviewed in [1–3]). The ATM protein is a member of the phosphatidyl inositol 3 kinase-like protein kinase (PIKK) family of serine/threonine

Abbreviations: A-T, ataxia telangiectasia; ATM, ataxia-telangiectasia mutated; BSA, bovine serum albumin; DMSO, dimethyl sulphoxide; DNA-PK, DNA-dependent protein kinase; DSB, double-strand break; IR, ionizing radiation; NCS, neocarzinostatin; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PIKK, phosphatidyl inositol 3 kinase-like protein kinase; SDS, sodium dodecyl sulphate; topo I, topoisomerase I; topo II, topoisomerase II; UV, ultraviolet radiation

* Corresponding author. Tel.: +1-403-220-7628; fax: +1-403-210-3899.
E-mail address: leesmill@ucalgary.ca (S.P. Lees-Miller).
[1] Present address: Department of Medical Biochemistry, Kurume University School of Medicine, 67 Asahimachi, Kurume 830-0011, Japan.

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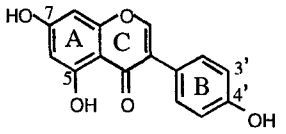
protein kinases and phosphorylates substrates on serines or threonines that are followed by glutamine (S/T-Q motifs) [4,5]. The protein kinase activity of ATM increases 2–3-fold when cells are exposed to DNA-damaging agents such as ionizing radiation (IR) or the radiomimetic agent, neocarzinostatin (NCS) [6,7]. Activation of ATM is triggered by autophosphorylation on serine 1981 and conversion of ATM from an inactive dimeric or multimeric form to an active, monomeric form [8]. Once activated, ATM phosphorylates downstream target proteins resulting in activation of cell cycle checkpoints, arrest at G_1/S, S and G_2/M phases of the cell cycle and coordination of the cellular response to DNA double-strand breaks (DSBs) (reviewed in [3]).

One of the most well characterized ATM substrates is the p53 tumour suppressor protein (reviewed in [9–12]). ATM phosphorylates p53 on serine 15 in vitro [6,7,13–15], and IR-induced accumulation and phosphorylation of serine 15 is delayed and attenuated in ATM-deficient cells, suggesting that ATM directly phosphorylates serine 15 of p53 in response to IR [7,13,16]. IR also induces phosphorylation of p53 on serines 6, 9, 20, 33, 46, 315, and 392, and phosphorylation on serines 9, 15, 20, and 46 is defective in ATM-deficient cells [17]; however it is not known whether ATM acts on these sites directly or indirectly. Other DNA damage-inducing agents such as ultraviolet radiation (UV), adriamycin and nitric oxide, also induce multisite phosphorylation of p53 [17–19]. Nitric oxide-induced phosphorylation of p53 on serine 15 is partially ATM-dependent, while UV-induced p53 phosphorylation is not ATM-dependent [18,19]. Whether ATM is required for adriamycin-induced p53 phosphorylation has not yet been reported. Amino-terminal phosphorylation of p53 promotes interactions with acetyl transferases that in turn acetylate p53, promote sequence-specific DNA-binding of p53 and contribute to DNA-damage-induced activation of p53 (reviewed in [9–12]). Once activated, p53 *trans*-activates genes required for regulation of the cell cycle and/or apoptosis.

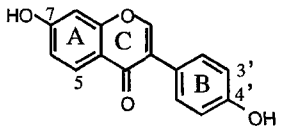
IR also induces phosphorylation of histone H2AX on serine 139 at sites of DNA damage; indeed, phosphorylation of histone H2AX is widely accepted as an indicator of the presence of DSBs [20–22]. ATM is responsible for the majority of IR-induced serine 139 phosphorylation of histone H2AX, although the related protein kinase, DNA-dependent protein kinase (DNA-PKcs), may play a minor role [23]. Histone H2AX phosphorylation is also induced by NCS, bleomycin and the topoisomerase (topo) II inhibitor, etoposide, but not by UV [23].

We previously showed that incubation of cells with the bioflavonoid genistein stimulated the protein kinase activity of ATM, induced an ATM-dependent increase in p53 protein levels, and increased phosphorylation of p53 at serine 15 and the binding of p53 to its cognate DNA-binding sequence [24]. Genistein is commonly used as a protein tyrosine kinase inhibitor [25]; however, it also inhibits topo I [26] and topo II [26–29]. Genistein also inhibits cell proliferation [30,31], induces cell cycle arrest [32], induces apoptosis [33], and radiosensitizes cells [34]. Taken together, these observations suggest that genistein induces DNA damage and the activation of DNA damage response pathways.

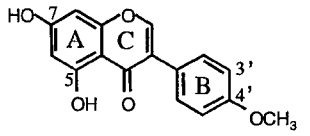
Genistein: (4',5,7-trihydroxyisoflavone)



Daidzein: (4',7-dihydroxyisoflavone)



Biochanin A: (5, 7-dihydroxy-4'-methoxyisoflavone)



Quercetin: (3,3',4',5,7-pentahydroxyflavone)

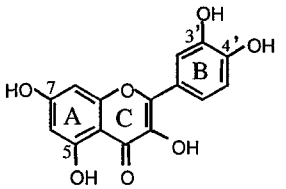


Fig. 1. The structures of the bioflavonoids genistein, daidzein, biochanin A, and quercetin.

Here, we have examined the effects of genistein and the related bioflavonoids, daidzein, biochanin A, and quercetin, on ATM-dependent DNA damage response pathways. Genistein, daidzein and biochanin A are all isoflavones, but differ in the substitution of the A and B rings (Fig. 1). Despite these structural similarities, only genistein is a topoisomerase poison, inhibiting topo I and topo II [26,27,35]. Quercetin is a flavonol that is hydroxylated at positions 3, 3′, 4′, 5, and 7 (Fig. 1). Like genistein, quercetin inhibits topo I and topo II [27,36], and is a protein kinase inhibitor [37,38]. We find that genistein and quercetin, but not biochanin A or daidzein induce phosphorylation of ATM on serine 1981, phosphorylation of histone H2AX on serine 139, and accumulation and multisite phosphorylation of p53. Significantly, ATM was required for genistein-induced p53 accumulation and phosphorylation but not for quercetin-induced accumulation and phosphorylation of p53. Our results suggest that genistein and quercetin activate novel as well as overlapping DNA damage response pathways.

2. Materials and methods

2.1. Reagents

Daidzein (4′,7-dihydroxyisoflavone), biochanin A (5,7-dihydroxy-4′-methoxyisoflavone), quercetin (3,3′,4′,5,7-pentahydroxyflavone), and genistein (4′,5,7-trihydroxyisoflavone) were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of the flavonoids were prepared in dimethyl sulphoxide (DMSO), protected from light, and stored at −20 °C.

2.2. Cells

ATM-proficient (BT and C3ABR) and ATM-deficient (L3 and AT1ABR) human lymphoblastoid cell lines were described [24,39,40]. The L3 cell line contains a homozygous mutation at codon 103 of *ATM*, and only 34 amino acids of the 369 kDa ATM protein are expressed [40]. AT1ABR cells express ATM protein that contains a three amino acid deletion outside the ATM kinase domain which results in an unstable protein and loss of protein kinase activity [39,40]. Cells were maintained as suspension cultures in either RPMI 1640 (BT and L3) or DMEM/F12 (C3ABR and AT1ABR) media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 50 U/ml penicillin G and 50 μg/ml streptomycin sulfate. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Flavonoids, or an equivalent volume of carrier (DMSO), were added directly to the cell media at the start of each experiment.

2.3. Antibodies

DO-1 (a monoclonal antibody to p53), agarose conjugated DO-1, agarose-conjugated PAb1801, and antibody H-124 to histone H2A were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphospecific antibodies to serines 6, 9, 15, 20, 46, and 392 of p53 were purchased from Cell Signaling Technology (New England Biolabs, Beverly, MA). Phosphospecific antibodies to serine 139 of histone H2AX and serine 1981 of ATM were purchased from Upstate (Lake Placid, NY) and Rockland Immunochemicals (Gilbertsville, PA), respectively. Antibodies to actin were from Sigma-Aldrich. A polyclonal antibody (DPK1) to the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) and antibody 4BA to ATM were as described previously [24,41].

2.4. Immunodetection

2.4.1. p53

Unless otherwise indicated, p53 was immunoprecipitated from whole cell extracts of detergent lysed cells and then probed with phosphospecific antibodies as described [17]. Briefly, cells were harvested by centrifugation, washed in phosphate buffered saline (PBS), and then lysed in NET-T buffer [150 mM NaCl, 5 mM EDTA, 50 mM Tris–HCl pH 7.5, 1% (v/v) Triton X-100] containing phosphatase and protease inhibitors (50 mM NaF, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 μg/ml pepstatin, and 0.5 mM phenyl methylsulphonylfluoride). Samples were placed on ice, sonicated three times (for 10 s each time), then centrifuged at 10,000 × *g* for 20 min at 4 °C. Protein concentrations were determined using the Detergent Compatible Protein Assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions, using bovine serum albumin (BSA) as the standard. For analysis of phosphorylation, p53 was immunoprecipitated from 1 mg of whole cell extract (per site analyzed) using 2.5 μg each of agarose-conjugated DO-1 and PAb1801 antibodies. Immunoprecipitates were washed five times in NET-T buffer (1 ml per wash) then resuspended in sodium dodecyl sulphate (SDS) sample buffer, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on 10% polyacrylamide gels. Gels were transferred to nitrocellulose membranes in electroblot buffer [25 mM Tris–base, 192 mM glycine, 20% (v/v) methanol] at 100 V for 60 min, then incubated with phosphospecific antisera to individual p53 sites as indicated. Immunoblots were developed by ECL detection (Amersham Biosciences, Baie d'Urfe, PQ) according to the manufacturer's instructions. Immunoblots were then stripped and reprobed with DO-1 for determination of total p53 levels. In all experiments, immunoblots from ATM-proficient and deficient cells were always probed and developed together for exactly the same length of time. Where indicated, autoradiographs of ECL-treated immunoblots were scanned and quantitated using ImageQuant software (Amersham Biosciences).

In order to confirm that phosphospecific antibodies were indeed specific, antibodies to p53 phosphorylated at serines 6, 9, 15, 20, 46, and 392 were pre-incubated with the corresponding nonphospho- or phospho-p53 peptide prior to incubation with the immunoblot, and probed against p53 from untreated, IR- or genistein-treated cells. In every case, addition of phosphopeptide ablated the signal while addition of the non-phosphopeptide had no discernable effect (data not shown).

2.4.2. ATM

Cells were harvested, washed in PBS, then lysed in NET-T buffer exactly as described for p53. Sixty micrograms of total protein were separated on 8% acrylamide SDS gels, transferred to nitrocellulose in SDS-electroblot buffer [25 mM Tris–base, 192 mM glycine, 0.04% (w/v) SDS, 20% (v/v) methanol] at 100 V for 60 min, and then analyzed by immunoblot using a phosphospecific antibody to serine 1981 of ATM. After development with ECL reagent, blots were stripped and probed with polyclonal antibody 4BA to ATM, followed by polyclonal antibody DPK1 to DNA-PKcs.

2.4.3. H2AX

Cells were harvested, washed in PBS followed by hypotonic buffer and lysed by freeze-thaw as described previously [24]. The supernatant (S10) was removed and the pellet was resuspended in 0.2 M sulphuric acid, incubated on ice for 30 min, and then centrifuged at 11,000 × *g* for 10 min at 4 °C. Supernatants were dialyzed against two changes of 0.1 M acetic acid, followed by three changes of ddH_2O. Protein concentrations were determined using the Detergent Compatible Assay (Biorad) as described above. Forty micrograms of protein were analyzed by SDS-PAGE on 16% acrylamide gels. Proteins were transferred to nitrocellulose as described for p53. Immunoblots were probed first for phosphorylation of histone H2AX on serine 139, then membranes were stripped and reprobed with an antibody to total histone H2A.

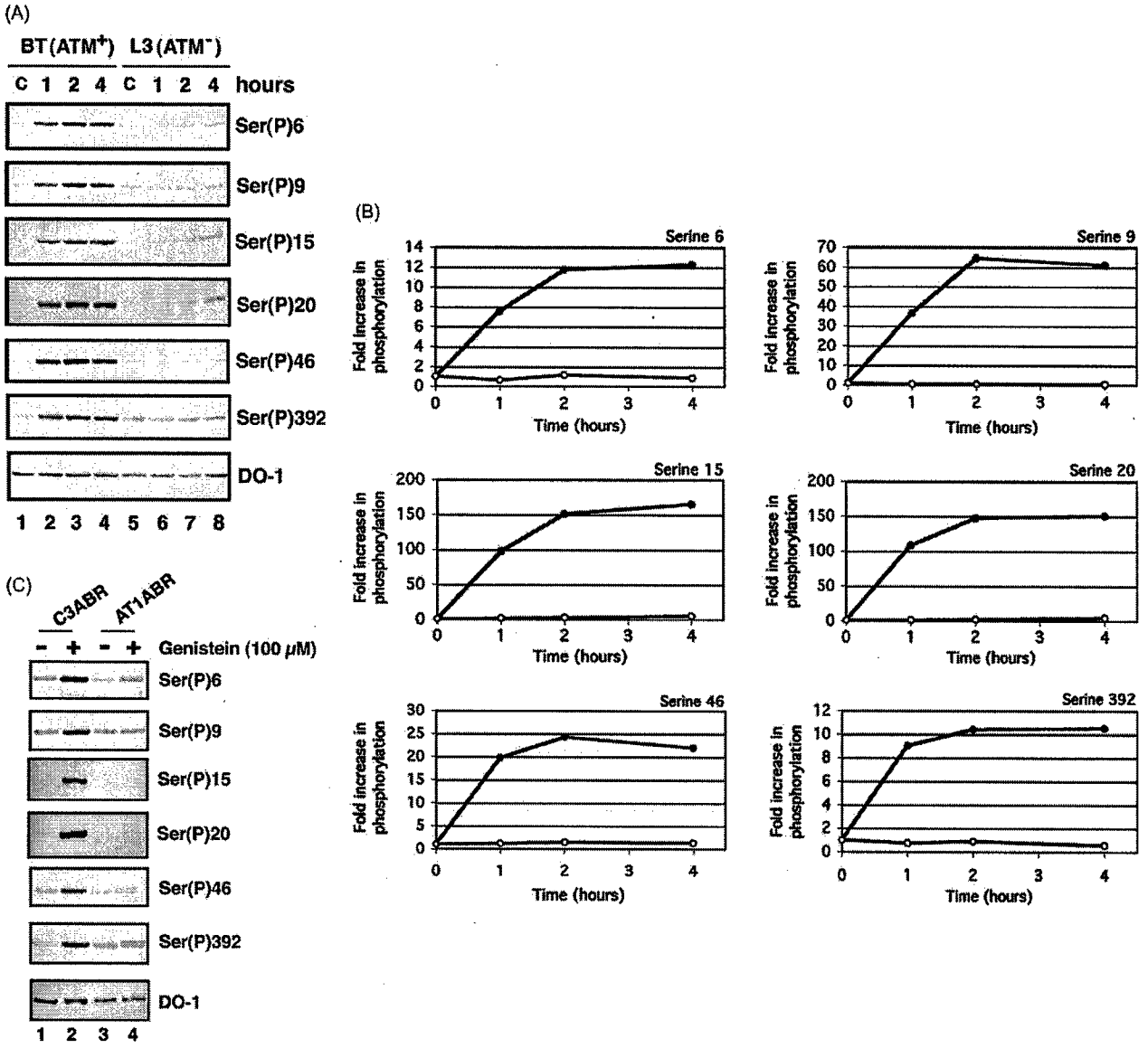


Fig. 2. Genistein-induced accumulation of p53 and p53 phosphorylation on serines 6, 9, 15, 20, 46, and 392 is defective in ATM-deficient human cell lines. Panel A: ATM-proficient (BT) or ATM-deficient cells (L3) were either mock treated with DMSO (C, lanes 1 and 5) or treated with 100 μM genistein for 1, 2, or 4 h, as indicated (lanes 2–4 and 6–8). For each phosphorylation site analyzed, p53 was immunoprecipitated from 1 mg of whole cell extract from detergent-lysed cells as described in Section 2. Immunoprecipitates were analyzed by SDS-PAGE, and blots were probed with phosphospecific antisera to serines 6, 9, 15, 20, 46 or 392, followed by incubation with monoclonal antibody DO-1 for total p53 as indicated. Panel B: The immunoblots shown in panel A were scanned, quantitated and p53 phosphorylation was normalized to total p53 (as estimated by DO-1 immunoreactivity). Results are expressed as fold increase in the treated samples over the untreated samples. Closed circles indicate BT cells and open circles represent L3 cells. Panel C: C3ABR and AT1ABR cells were incubated with 100 μM genistein for 2 h as indicated. p53 was immunoprecipitated and analyzed for phosphorylation exactly as described in panel A.

3. Results

3.1. Genistein induces ATM-dependent phosphorylation of p53 at serines 6, 9, 15, 20, 46, and 392

p53 was immunoprecipitated from detergent lysates of BT (ATM-positive) and L3 (ATM-deficient) cells using agarose-conjugated DO-1 and PAb1801 monoclonal antibodies and immunoblots were probed with phosphospecific antibodies to p53 as described in Section 2. Genistein induced phosphorylation of p53 on serines 6, 9, 15, 20, 46, and 392 in the ATM-proficient cell line, BT (Fig. 2A). Phosphorylation occurred within 1 h of exposure to genistein and persisted for at least 4 h (Fig. 2A). However, both accumulation and phosphorylation of p53 were defective in the ATM-deficient cell line, L3 (Fig. 2A and B). Very similar results were obtained in C3ABR (ATM-proficient) and AT1ABR (ATM-defective) cells (Fig. 2C). We conclude that genistein induces multisite phosphorylation of p53 on serines 6, 9, 15, 20, 46, and 392 and that p53 accumulation and/or phosphorylation at each site requires the presence of ATM.

3.2. Genistein induces phosphorylation of ATM at serine 1981

To determine if genistein also induced phosphorylation of ATM, extracts were prepared from genistein-treated cells and probed for phosphorylation at serine 1981. Exposure to 100 μM genistein induced robust phosphorylation of ATM at serine 1981 (Fig. 3A), and phosphorylation was detectable 2 h after treatment with 10 μM genistein (Fig. 3B).

3.3. Genistein induces phosphorylation of histone H2AX

Before examining the effects of genistein on histone H2AX phosphorylation, we first examined the effects of IR. BT cells (ATM-proficient) were irradiated (10 Gy) and histones were extracted as described in Section 2. IR induced phosphorylation of H2AX at serine 139 and reached maximal levels at 30–60 min (Fig. 4A). IR did not induce histone H2AX phosphorylation in the ATM-deficient cell line, L3 (data not shown). We next treated BT and L3 cells with 100 μM genistein for 0, 1, or 2 h. Genistein-induced phosphorylation of histone H2AX at serine 139 was observed in the ATM-positive cell line, BT, but not in the ATM negative cell line, L3 (Fig. 4B), suggesting that genistein induces DNA damage in the form of DSBs and that ATM is required for phosphorylation of histone H2AX in response to the induced damage.

3.4. Quercetin but not daidzein or biochanin A induces autophosphorylation of ATM on serine 1981 and phosphorylation of p53 on serines 15 and 20

To determine whether the related flavonoids also induced p53 phosphorylation, BT cells (ATM-proficient) were incubated with DMSO, or 100 μM daidzein, biochanin A, or quercetin for 2 h. Nuclear extracts (P10) were prepared as described previously [24] and examined by immunoblot to detect phosphorylation of p53 on serine 15 or serine 20 (Fig. 5). Although the chemical structures of daidzein and biochanin A are very similar to that of genistein (Fig. 1), neither daidzein nor biochanin A induced a significant increase in p53 protein levels, as judged by reactivity with DO-1, nor did they induce phosphorylation of p53 at serine 15 or serine 20 (Fig. 5, lanes 1–3). In contrast, exposure of cells to quercetin resulted in increased p53 protein levels and increased phosphorylation of p53 on serine 15 and serine 20 (Fig. 5, lane 4).

We therefore examined whether daidzein, biochanin A and quercetin induced phosphorylation of ATM on serine 1981. BT cells (ATM-proficient) were incubated with 100 μM quercetin, daidzein or biochanin A and harvested after 1, 2 or 4 h. Under these conditions, quercetin induced phosphorylation of ATM on serine 1981 (Fig. 6), whereas biochanin A and daidzein did not (data not shown).

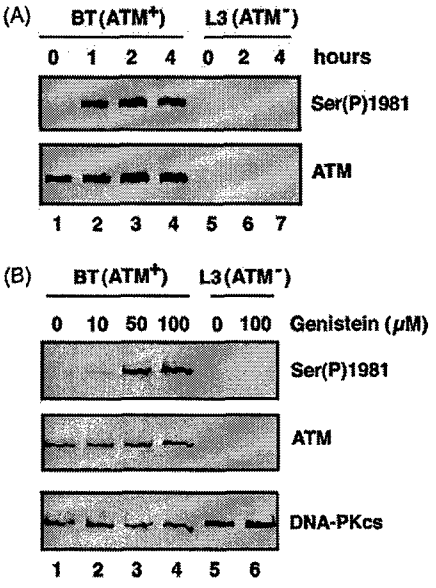


Fig. 3. Genistein induces phosphorylation of ATM on serine 1981: Panel A: ATM-proficient (BT) or ATM-deficient (L3) cells were incubated with DMSO (lanes 1 and 5) or 100 μM genistein for 1, 2, or 4 h, as indicated. One hundred microgram of protein were analyzed by SDS-PAGE and probed with a phosphospecific antibody to serine 1981 followed by a polyclonal antibody to ATM, as indicated. Panel B: BT or L3 cells were incubated with DMSO (lane 1 and 5) or 10, 50 or 100 μM genistein, as indicated. After 2 h, cells were harvested and assayed for phosphorylation of ATM as described in panel A. To confirm the presence of equal amounts of protein in the L3 lanes, immunoblots were also probed for the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) using the DPK1 antibody.

3.5. Quercetin-induced phosphorylation of p53 does not require ATM

To determine whether quercetin induced phosphorylation of p53 at multiple sites, and whether quercetin-induced

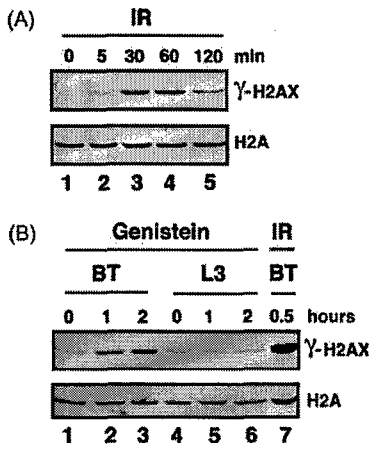


Fig. 4. IR and genistein induce ATM-dependent phosphorylation of H2AX: Panel A: ATM-proficient cells (BT) were irradiated with 10 Gy and harvested after 5, 30, 60 or 120 min, as indicated. Histones were extracted from the nuclear pellet as described in Section 2, and 40 μg of protein were analyzed on 16% acrylamide SDS gels, transferred to nitrocellulose and probed with a phosphospecific antibody to serine 139 of histone H2AX (upper panel). The immunoblot was then stripped and probed for histone H2A (lower panel). H2AX phosphorylation was not observed in L3 cells that had been exposed to IR under similar conditions (data not shown). Panel B: ATM-proficient cells (BT) (lanes 1–3) or ATM-deficient cells (L3) (lanes 4–6) were either mock treated with DMSO (lanes 1 and 4), or incubated with genistein (100 μM) for 1 or 2 h, as indicated (lanes 2 and 3, and 5 and 6). The sample in lane 7 was from irradiated (10 Gy) BT cells and is shown for comparison. The immunoblot was probed with the phosphospecific antibody to serine 139 of histone H2AX (upper panel), then stripped and reprobed for histone H2A (lower panel).

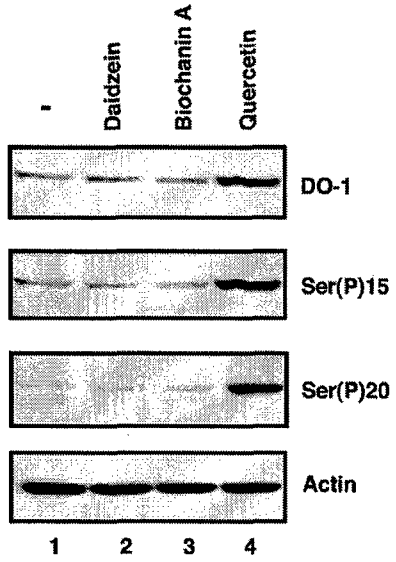


Fig. 5. Quercetin, but not daidzein or biochanin A, induces phosphorylation of p53 on serines 15 and 20: ATM-proficient cells (BT) were treated for 2 h with 100 μM daidzein (lane 2), biochanin A (lane 3), quercetin (lane 4), or an equivalent volume of DMSO (lane 1). Nuclear extracts (P10) were prepared as described previously [24] and 30 μg of protein were analyzed by SDS-PAGE followed by immunoblot analysis, as described in Section 2. Blots were probed first with phosphospecific antisera to serine 15 or serine 20, then stripped and probed with the pan-reactive p53 antibody, DO-1, or actin as indicated.

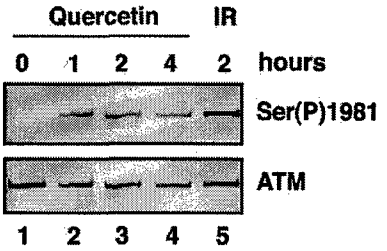


Fig. 6. Quercetin induces phosphorylation of ATM on serine 1981: BT cells were incubated with DMSO (0) or 100 μM quercetin for 1, 2 or 4 h, as indicated. Extracts were prepared and analyzed for ATM phosphorylation (upper panel) followed by total ATM (lower panel) as described in Section 2.

phosphorylation of p53 was ATM-dependent, BT (ATM-proficient) and L3 (ATM-deficient) cells were incubated with 100 μM quercetin for 1, 2 or 4 h (Fig. 7A). p53 was immunoprecipitated and phosphorylation at serines 6, 9,15, 20, 46, and 392 was determined as described in Fig. 2. Like IR, genistein, adriamycin, and nitric oxide, quercetin induced phosphorylation of p53 at multiple sites, including serines 6, 9, 15, 20, 46, and 392 (Fig. 7A). However, in striking contrast to the results obtained with genistein, neither p53 accumulation nor p53 phosphorylation required the presence of the ATM protein (Fig. 7A and B). These results clearly show that ATM is not required for phosphorylation of p53 in response to quercetin.

3.6. Quercetin induces ATM-dependent phosphorylation of histone H2AX on serine 139

Since quercetin induced ATM phosphorylation at serine 1981, a site that is associated with activation of ATM [8], it was possible that quercetin could induce ATM-dependent phosphorylation of other substrates, for example, histone H2AX. BT cells were therefore treated with quercetin and analyzed for histone H2AX phosphorylation. Quercetin was found to induce ATM-dependent phosphorylation of histone H2AX on serine 139 (Fig. 8). Together, our data suggest that quercetin activates ATM-dependent and well as ATM-independent signaling pathways.

4. Discussion

ATM plays a major role in the cellular response to DNA DSBs. DNA damaging agents that produce DSBs, such as IR, induce activation of the protein kinase activity of ATM, and phosphorylation of ATM on serine 1981 [8]. IR, bleomycin, etoposide and NCS [23] also induce phosphorylation of histone H2AX, which is regarded as a sensitive indicator of the presence of chromatin-associated DSBs [20,43]. However, hypotonic buffer and the topo II catalytic inhibitor chloroquine, which are not expected to produce DSBs and do not induce phosphorylation of histone H2AX, also induce ATM serine 1981 phosphorylation, suggesting

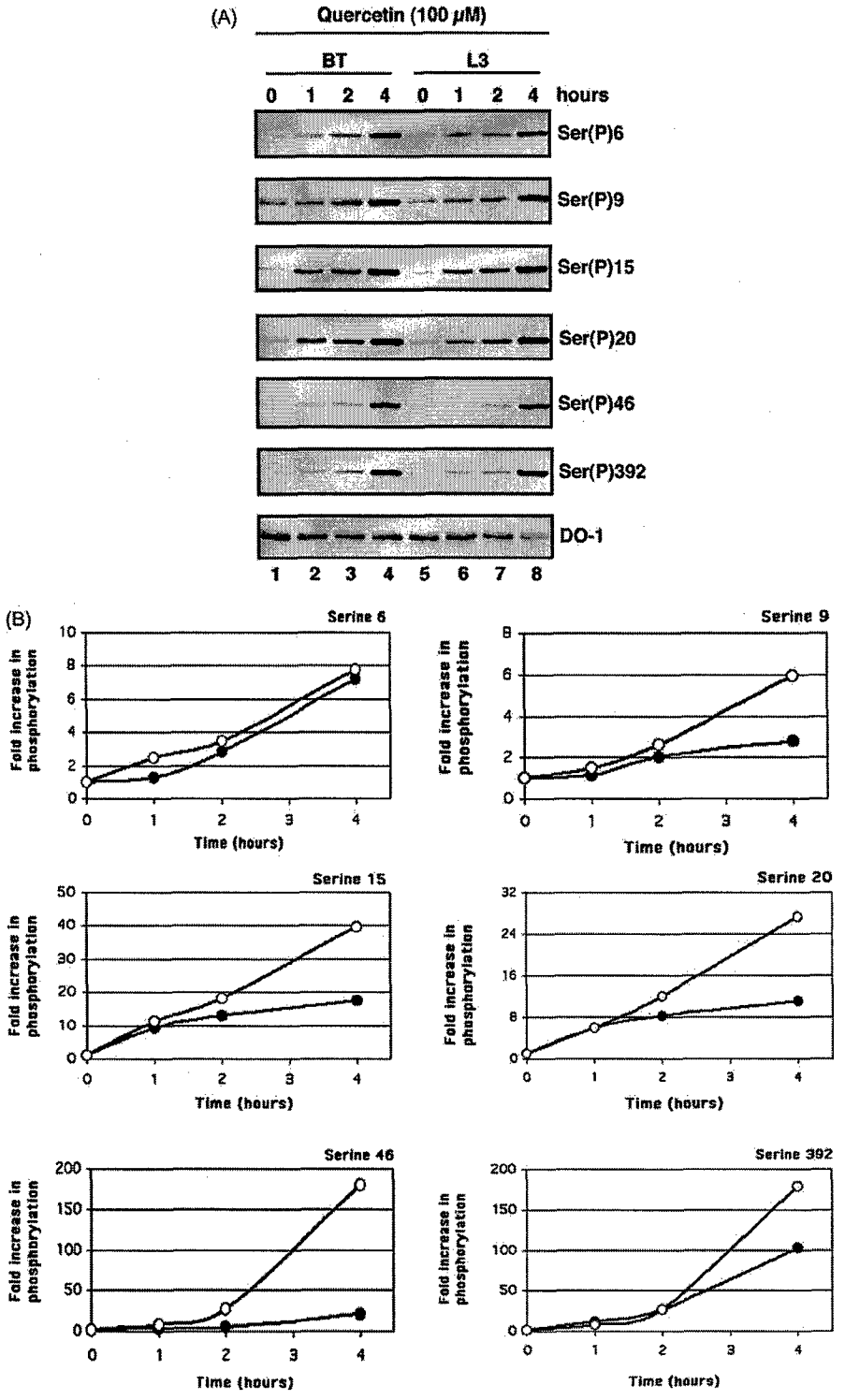


Fig. 7. Quercetin-induces ATM-independent accumulation and multisite phosphorylation of p53: Panel A: ATM-proficient (BT) or ATM-defective cells (L3) were treated with DMSO (0), or 100 μM quercetin for 1, 2, or 4 h. p53 was immunoprecipitated and analyzed for phosphorylation of p53 at serines 6, 9, 15, 20, 46, or 392, or total p53 (DO-1), as described in Fig. 2. Panel B: Results in panel A were quantitated as described for Fig. 2. Closed circles indicate BT cells and open circles represent L3 cells.

Table 1
Summary of effects of various DNA damaging agents on phosphorylation of ATM, histone H2AX and p53

DNA damaging agent	H2AX phosphorylation (Ser139)	ATM phosphorylation (Ser1981)	p53 phosphorylation
IR	Yes [23]	Yes [8]	Multiple sites; serines 9, 15, 20, 46 ATM-dependent [17,19]
UV	No [23]	No [8]	Multiple sites; ATM-independent [17,19]
NCS	Yes [23]	Yes [42]	Serine 15, ATM-dependent [6,42]
Adriamycin	Nd	Nd	Multiple sites [19]
Nitric oxide	Nd	Nd	Multiple sites, partially ATM-dependent [18]
Etoposide	Yes [23]	Nd	Serine 15, ATM-independent [24]
Bleomycin	Yes [23]	Nd	Nd
Genistein	Yes (this study)	Yes (this study)	Multiple sites, serines 6, 9, 15, 20, 46, 392 ATM-dependent (this study)
Quercetin	Yes (this study)	Yes (this study)	Multiple sites; ATM-independent (this study)

Nd = not determined. See text for details.

that changes in chromatin structure can also induce ATM activation [8]. Once activated, ATM phosphorylates downstream targets that activate cell cycle checkpoints to induce cell cycle arrest at G_1/S, S and G_2/M.

Two of the major targets of ATM are p53 and histone H2AX. IR induces ATM-dependent phosphorylation of p53 on serines 9, 15, 20, and 46, ATM-independent phosphorylation of p53 on serines 6, 33 and 392, and ATM-dependent phosphorylation of histone H2AX on serine 139. NCS also induces activation of the protein kinase activity of ATM, phosphorylation of ATM on serine 1981, and ATM-dependent phosphorylation of p53 on serine 15 [6,42]. NCS-mediated phosphorylation of p53 at additional sites has not been examined. Other DNA damaging agents such as UV, adriamycin, and nitric oxide also induce multisite phosphorylation of p53, however, in the case of UV, which induces bulky lesions but not DSBs, phosphorylation of p53 is ATM-independent [17–19]. Accordingly, UV does not induce serine 139 phosphorylation of histone H2AX [23]. Thus, analysis of the phosphorylation status of ATM, p53, and histone H2AX can distinguish ATM-dependent and ATM-independent DNA damage response pathways (summarized in Table 1).

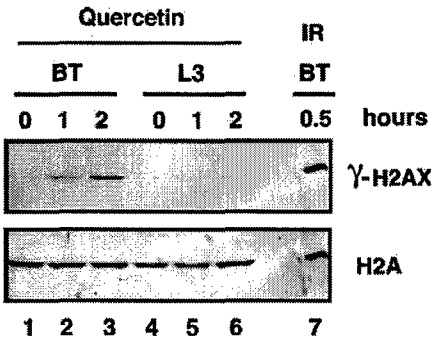


Fig. 8. Quercetin induces ATM-dependent phosphorylation of histone H2AX on serine 139: ATM-proficient (BT) or ATM-deficient (L3) cells were either mock treated with DMSO or incubated with 100 μM quercetin for 1, 2 or 4 h, then processed for histone H2AX phosphorylation as described in Section 2. The sample in lane 7 was prepared from BT cells that had been irradiated with 10 Gy and is shown for comparison. The upper panel shows histone H2AX phosphorylation on serine 139; the lower panel shows histone H2A.

Genistein and quercetin both act as topo I and II poisons and are therefore expected to produce DNA DSBs. Indeed, genistein has been shown to induce DNA fragmentation in cells [44]. Here, we show that genistein and quercetin both induce ATM phosphorylation on serine 1981 and histone H2AX phosphorylation on serine 139, suggesting that both compounds induce DNA DSBs leading to autophosphorylation of ATM and activation of its protein kinase activity. In contrast, daidzein, and biochanin A, which do not inhibit topoisomerases and are not expected to induce DSBs, did not significantly induce phosphorylation of either ATM or p53. We speculate that the type of DNA lesions produced by genistein and quercetin and/or the mechanisms to detect this damage must be subtly different, as the ability of genistein and quercetin to induce ATM-independent phosphorylation of p53 was dramatically different. It is interesting to note that both genistein and quercetin also inhibit protein kinases, and thus we cannot eliminate the possibility that this function contributes to their ability to activate DNA damage response pathways. We propose that genistein and quercetin may be useful tools to distinguish ATM-dependent and ATM-independent DNA damage response pathways that could help identify the particular type of DNA damage and or chromatin changes that are responsible for activation of ATM and ATM-dependent downstream signaling pathways.

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Review

DNA damage-induced activation of ATM and ATM-dependent signaling pathways

Ebba U. Kurz, Susan P. Lees-Miller*

Cancer Biology Research Group, Department of Biochemistry and Molecular Biology, University of Calgary, 3330 Hospital Drive NW, Calgary, AB, Canada T2N 4N1

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Abstract

Ataxia-telangiectasia mutated (ATM) plays a key role in regulating the cellular response to ionizing radiation. Activation of ATM results in phosphorylation of many downstream targets that modulate numerous damage response pathways, most notably cell cycle checkpoints. In this review, we describe recent developments in our understanding of the mechanism of activation of ATM and its downstream signaling pathways, and explore whether DNA double-strand breaks are the sole activators of ATM and ATM-dependent signaling pathways.

Keywords: Ataxia-telangiectasia mutated; Cell cycle checkpoint; DNA damage; DNA double-strand breaks; Ionizing radiation; p53

1. Introduction

Ataxia-telangiectasia (A-T) is a rare human disease characterized by cerebellar degeneration, immune system defects and cancer predisposition [1–3]. The disease has been the subject of intense scientific scrutiny, particularly since the identification in 1995 of the gene mutated in A-T, *ATM* [4,5]. The ataxia-telangiectasia mutated protein (ATM) has emerged as a central player in the cellular response to ionizing radiation (IR), in which it plays a critical role in the activation of cell cycle checkpoints that lead to DNA damage-induced arrest at G_1/S, S, and G_2/M. The many properties of ATM have been the subject of several recent reviews [1,2,6,7]. Here, we will highlight some of the new developments in the field and address some of the prominent, unanswered questions related to ATM structure and function.

2. The ATM protein

ATM is a member of the phosphatidylinositol 3-kinase-like family of serine/threonine protein kinases (PIKKs). Other members of this protein family include ATM- and Rad3-related (ATR), DNA-dependent protein kinase catalytic subunit (DNA-PKcs), mammalian target of rapamycin (mTOR), and ATX/hSMG-1 [1,7]. The PIKKs represent a subclass of "atypical" protein kinases within the overall eukaryotic protein kinase family [8]. ATM, like other members of this protein kinase family, phosphorylates its substrates on serine or threonine that is followed by glutamine, i.e. an SQ or TQ motif [9,10]. ATM shares several features with other members of the PIKK family, including a FAT domain (named due to amino acid conservation between the PIKK family members *F*RAP, *A*TR, and *T*RRAP), a phosphoinositide 3,4-kinase (PI3K) domain and a FAT carboxy-terminal (FAT-C) domain [11] (Fig. 1). One function of the FAT domain may be to interact with the kinase domain to stabilize the carboxy-terminal region of the protein [12]. Bioinformatics analysis indicates that the amino-terminal portions of ATM, ATR, mTOR and, likely, DNA-PKcs are composed of multiple *h*untingtin, *e*longation factor 3, *A* subunit of protein phosphatase 2A and *T*OR1 (HEAT) repeats [13]. Each HEAT repeat is composed of two interacting, anti-parallel alpha helices linked by a flexible loop. The amino-terminal regions of the PIKK proteins, therefore, are predicted to consist of multiple, interacting

Abbreviations: A-T, ataxia-telangiectasia; ATM, ataxia-telangiectasia mutated; ATR, ATM- and Rad3-related; ds, double-stranded; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; DSB, DNA double-strand break; HEAT, huntingtin, elongation factor 3, A subunit of protein phosphatase 2A and TOR1; IKK, IκB kinase; IP, immunoprecipitation; IR, ionizing radiation; mTOR, mammalian target of rapamycin; NCS, neocarzinostatin; PI3K, phosphoinositide 3,4-kinase; PIKK, phosphatidylinositol 3-kinase-like protein kinase; ROS, reactive oxygen species; SSB, DNA single-strand break; topo, topoisomerase

* Corresponding author. Tel.: +1-403-220-7628; fax: +1-403-210-3899.
E-mail address: leesmill@ucalgary.ca (S.P. Lees-Miller).

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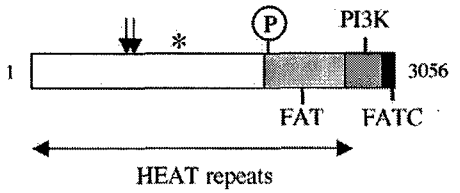

Fig. 1. Schematic of the human ATM polypeptide. The amino acid sequence of human ATM (Genbank accession number Q13315) was obtained from the NCBI database and the domain boundaries of the FAT, phosphoinositide 3,4-kinase, and FAT-C domains were identified using the Smartv3.5/Pfamv9.0 algorithm [99]. Human ATM is a 3056 amino acid polypeptide. The amino-terminal 2659 amino acids of ATM contain 49 HEAT repeats that are predicted to form a massive alpha helical scaffold [13]. The FAT domain is located between amino acids 1966 and 2565 of ATM. The PI3K domain is located between amino acids 2609 and 2976 and the FAT-C domain between amino acids 3024 and 3056 [11]. The autophosphorylation site, serine 1981 [12], indicated by the P, is located in a small linker region between HEAT repeats 35 and 36, in the amino-terminal region of the FAT domain. Two caspase 3 cleavage sites, indicated by arrows, have been identified at amino acids 814–818 and 860–864 [100]. A putative leucine zipper region, indicated by the asterisk, resides between amino acids 1216 and 1241 [101].

HEAT repeats that form a massive, superhelical scaffolding matrix [13]. While the function of the HEAT repeat domain remains unknown, we speculate that it could provide a surface for interactions with other proteins.

The first indications of the overall shape and dimensions of the ATM protein have recently emerged. Single particle electron microscopic analysis reveals that ATM has a large "head" domain of approximately 115 Å by 75–140 Å, as well as a long "arm" structure that protrudes from the head region [14]. The overall shape of ATM is, thus, similar to that of the related protein, DNA-PKcs, at similar resolution [15]. Both proteins have the ability to interact with ends of double-stranded (ds) DNA [16–19], and this interaction may be facilitated by a conformational change that causes the arm region to wrap around the DNA [14]. From the low-resolution (~30 Å) structure of ATM, we speculate that the HEAT domain corresponds to the head structure and the carboxy-terminal kinase domain to the arm. However, elucidation of the precise molecular architecture of ATM will require a higher resolution structure.

3. Activation of ATM in response to DNA damage

Perhaps one of the most important recent developments in the field has been insight into the mechanism of ATM activation in response to DNA damage. ATM is a predominantly nuclear protein, the levels of which do not change when cells are exposed to IR [20–23]. However, the protein kinase activity of ATM, as determined using immunoprecipitation (IP) kinase assays, increases two- to three-fold following cellular exposure to IR [22] or the radiomimetic neocarzinostatin (NCS) [20]. IR also induces increased incorporation of phosphate into ATM [12,24]. DNA damage-induced phosphate incorporation was not observed in cells expressing kinase-dead ATM, suggesting that IR induces autophosphorylation of ATM. Indeed, serine 1981, an SQ site located in the amino-terminal region of the FAT domain (Fig. 1), is rapidly phosphorylated in cells that have been exposed to even very low doses of IR [12]. ATM containing a serine to alanine mutation at amino acid 1981 failed to support IR-induced phosphorylation of p53 or cell cycle arrest, suggesting that serine 1981 phosphorylation is critical for the function of ATM [12]. *trans*-Phosphorylation and dissociation of ATM from an inactive dimeric (or higher order) form to an active monomeric form has, thus, emerged as one of the earliest events in the activation of ATM [12]. While phosphorylation of serine 1981 is certainly critical to the activation of ATM, it is also possible that ATM undergoes autophosphorylation at other sites important for the DNA damage response [24].

4. Does ATM detect DNA damage directly or indirectly?

Although these elegant experiments place ATM autophosphorylation as an important upstream event in the activation pathway, they do not address whether ATM is the primary sensor of DNA damage. If it were a direct sensor of DNA damage, ATM would be expected to interact directly with the damaged DNA. Addition of dsDNA does not stimulate ATM protein kinase activity in IP kinase assays [20], although addition of DNA does stimulate the activity of the purified ATM protein under some conditions in vitro [17,25]. Purified ATM binds ends of dsDNA in vitro [17], but the affinity of this interaction is not known. In crude cell extracts, ATM binds preferentially to irradiated DNA cellulose resin compared to undamaged DNA cellulose suggesting that ATM interacts with, or is recruited to, damaged DNA [26]. DNA damage also causes a portion of the total ATM to associate with a chromatin fraction that is resistant to detergent extraction [27], and serine 1981-phosphorylated ATM localizes to nuclear foci in response to DNA damage [12]. Together, these studies suggest that ATM may interact with, or be recruited to, DNA-damaged chromatin. However, these studies, carried out in intact cells or crude extracts, do not address whether ATM interacts with damaged DNA directly or indirectly.

The MRN complex (composed of Mre11, Rad50, and Nbs1 in humans, or xrs2 in yeast) fulfills many of the criteria for a DNA damage sensor [28,29]. Cells that are defective in Mre11 or Nbs1 have many features in common with A-T cells, including radiosensitivity and cell cycle checkpoint defects. Indeed, mutations in the human Mre11 gene are responsible for an A-T like disorder (ATLD), which clinically resembles A-T [30], suggesting that the MRN complex and ATM function in similar pathways in vivo. Indeed, several recent reports have placed the MRN complex as an upstream activator of ATM [31,32]. Autophosphorylation of ATM on serine 1981 is defective in cells that are compromised for

Nbs1 or Mre11 [31,32], and phosphorylation of some downstream targets of ATM is partially dependent on a functional MRN complex [31]. Intriguingly, the nuclease activity of Mre11 is required for activation of ATM, suggesting that DNA double-strand breaks (DSBs) may require processing prior to activation of ATM [31]. Also, ATM-mediated phosphorylation of Nbs1 on serine 343 occurs at DSBs, suggesting that the proteins co-localize at sites of DNA damage [33]. Together, these studies suggest an attractive model in which (i) the MRN complex binds directly to damaged DNA, (ii) Mre11 processes the DNA ends, (iii) ATM is recruited and activated through autophosphorylation and monomerization, and (iv) kinase-active, monomeric ATM phosphorylates substrates present at, or recruited to, the DSB (Fig. 2). Once activated, ATM may be released from the site of the DSB, enabling phosphorylation of more distal substrates, such as chromatin-bound histone H2AX (Fig. 2). This might account for the rapid DNA damage-induced phosphorylation of histone H2AX that occurs over several megabases surrounding the site of a DSB [34]. Indeed, the downstream checkpoint protein kinase Chk2 is recruited only transiently to sites of DNA damage [33]. It remains to be seen whether this is the only mechanism for activation of ATM by all DNA-damaging agents, or whether ATM can, under some circumstances, be activated by direct DNA binding, or by interaction with other DNA-binding partners. A very recent report suggests that the mediator of DNA damage checkpoint protein, MDC1 (also called NFBD1), which is known to interact with the MRN complex [35], is required for MRN-dependent activation of ATM at high doses of IR, whereas the p53-binding protein 53BP1 is required for activation of ATM at low doses of IR [36], suggesting that additional proteins may indeed regulate the activation of ATM.

Another fascinating clue to emerge in recent months is that the BRCA1 protein (reviewed by Ting and Lee, this issue) is required for IR-induced phosphorylation of some ATM substrates, including p53, c-jun, Nbs1, and Chk2 [37]. BRCA1 is also required for phosphorylation of p53 on serine 15 at later times after DNA damage in A-T cells, consistent with a requirement of BRCA1 for some ATR-mediated events [37]. In addition, BRCA1 is required for IR-induced phosphorylation of the structural maintenance of chromosomes protein, SMC1 [38], Chk1 [39], and, is itself a substrate of ATM [40]. However, BRCA1 is not required for the activation of ATM (as judged by ATM activity in IP kinase assays) [37]. DNA damage-induced phosphorylation of histone H2AX and human Rad17 (which recruits the Hus1–Rad1–Rad9 complex) also do not require the presence of BRCA1 [37]. Together, these data suggest that BRCA1 acts as a scaffolding protein that makes some, but not all, non-chromatin-associated substrates accessible to the activated ATM protein [37] (Fig. 2). BRCA1 has also been identified as part of a larger protein complex, BRCA1-associated genome surveillance complex (BASC), which contains ATM, the mismatch repair proteins MSH2, MSH6, MLH1, the Bloom syndrome helicase (BLM), the MRN complex, and replication factor C (RFC) [41], but how BASC functions in the DNA damage response remains to be determined. BRCA1 contains two carboxy-terminal BRCT repeats, motifs that have been shown recently to bind phosphoproteins [42–44]. It is, therefore, possible that phosphorylation of BRCA1 could regulate its association with partner proteins, and thus, further regulate phosphorylation of BRCA1-associated proteins by ATM. Interestingly, BRCA1 has also been shown to interact with protein phosphatase 1α [45], providing another possible mechanism for regulation.

As illustrated in Fig. 2, activation of ATM results in the phosphorylation of a plethora of downstream targets. Some of these proteins are direct substrates of ATM, for example, ATM likely directly phosphorylates serine 15 of p53 and serine 139 of histone H2AX in response to DNA damage. Others may be phosphorylated indirectly, through ATM-mediated regulation of other protein kinases, such as Chk1, Chk2 (reviewed in [1,7]), IκB kinase (IKK) [46], LKB1 [47], c-Abl [48], and the cyclin-dependent protein kinases (cdk1 and cdk2) (Fig. 2). It was previously thought that ATM signaled directly only to Chk2, while the related protein kinase, ATR, signaled to Chk1 (reviewed in [1,7]). Recent studies, however, have demonstrated that IR induces ATM-dependent phosphorylation of Chk1 on serine 317 [49] and serine 345 [47], suggesting that ATM and ATR-dependent signaling pathways could overlap rather than exist in parallel as originally proposed.

The most well characterized cellular response to activation of ATM is activation of G_1/S, intra-S, and G_2/M cell cycle checkpoints (Fig. 2). However, ATM also plays important roles in other aspects of the DNA damage response. One of the primary functions of cell cycle arrest may be to allow the cell more time to repair DNA damage. Thus, activation of ATM-dependent cell cycle checkpoints would be expected to engage mechanisms for the repair of IR-induced DNA damage. A very recent study [50], suggests that one way in which this occurs is via ATM-dependent, Chk2-mediated phosphorylation of BRCA1 on serine 988, and upregulation of DSB repair via homologous recombination [50,51] (Fig. 2). It is also possible that ATM-dependent activation of c-Abl [48,52] and c-Abl-induced phosphorylation of Rad51 on tyrosine 315 [53], play a role in regulation of DSB repair (Fig. 2). c-Abl also phosphorylates BRCA1 in an ATM-dependent manner [54], while BRCA1 interacts with BRCA2 which, in turn, interacts with Rad51 (reviewed in [55]). In addition, ATM also influences DNA damage-induced changes in gene expression through its effects on the IKK/NFκB pathway [46,56–58] and possibly c-jun [37] (Fig. 2). How ATM regulates DNA damage-induced apoptosis is still uncertain. One possibility is via p53-mediated upregulation of pro-apoptotic genes, such as Noxa and Puma [59] (Fig. 2). Given the importance of ATM in the cellular response to DSBs, it is perhaps surprising that ATM is not an essential gene in mammalian cells. This likely reflects the fact that other members of the PIKK family, such as ATR,

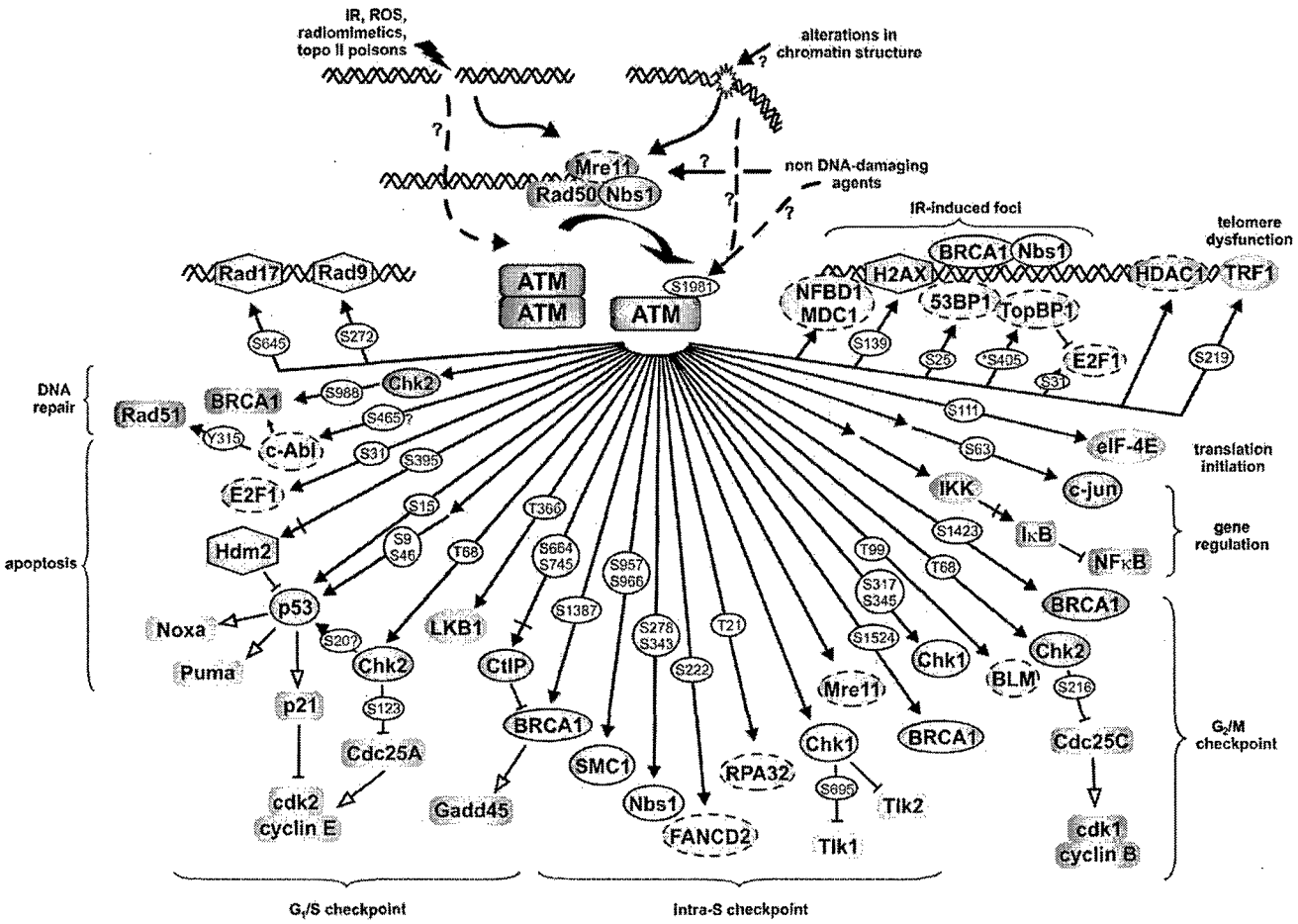

Fig. 2. A model for activation of ATM by DSBs and other forms of DNA damage. DSBs and possibly other forms of DNA damage, are detected by the MRN complex (composed of Mre11, Rad50, and Nbs1) [28,29,31,32]. It is not known whether MRN is required for activation of ATM in response to all DNA-damaging agents or whether ATM can, under some circumstances, be activated by mechanisms that do not involve DNA damage (represented by arrows with dashed lines). Of note, a recent study shows that NFBD1/MDC1 (which interacts with the MRN complex) and/or 53BP1 are also required for ATM activation under some conditions [36]. Activation of ATM occurs through its autophosphorylation on serine 1981 and conversion of ATM from an inactive dimer or higher order multimer, to an active, monomeric form [12]. Once activated, ATM can phosphorylate targets at the DSB or, possibly, be released to phosphorylate downstream targets. Some ATM-dependent phosphorylation events require the presence of BRCA1, which possibly acts as a scaffold, bringing substrates (represented by colored ovals with solid borders) to the activated ATM [37]. Substrates enclosed by dashed borders represent known ATM substrates that have been shown to associate with BRCA1, but for which a direct role for BRCA1 in ATM-mediated phosphorylation has not been demonstrated. BRCA1 is not required for ATM-mediated phosphorylation of Hdm2, Rad17, Rad9 or H2AX (indicated by hexagons with solid borders) [37]. ATM substrates for which the BRCA1 requirements are not known are shown as colored ovals without borders. Direct phosphorylation events are indicated by continuous black lines with solid arrowheads (→). Indirect phosphorylation events are indicated by the discontinuous lines with solid arrowheads (⇢). Phosphorylation sites, where known, are given in the white ovals. Downstream events that do not involve phosphorylation are indicated by lines with open arrowheads (—▷). Solid lines with bars indicate inhibition (—|). ATM-dependent phosphorylation events that relieve downstream inhibitions are designated by a line drawn perpendicularly through the continuous black lines with solid arrowheads (⊣→), for example, Hdm2 phosphorylation by ATM relieves its inhibition of p53. Proteins involved in DNA repair are indicated in dark blue, in apoptosis in green, in the G_1/S checkpoint in violet, in the intra S-phase checkpoint in yellow, in the G_2/M checkpoint in light blue, and in gene regulation/translation in beige. Proteins not belonging to any of these classifications are shown in grey. ATM substrates and phosphorylation sites are as described in previous reviews [1,7], or as indicated below: threonine 366 of LKB1 [47], serine 317 of Chk1 [49], serine 345 of Chk1 [47], serine 25 of 53BP1 [32], serine 405 of TopBP1 [102], threonine 21 of RPA32 [103], serine 63 of c-jun [37], serine 645 of Rad17 (phosphorylation of which is delayed in A-T cells) [37], and unidentified sites in NFBD1/MDC1 [35], HDAC [104], and IKK [46]. NCS-induced phosphorylation of Mre11 has been reported but its function is unknown [105]. BRCA1-mediated induction of Gadd45 is inferred from [106], however, a requirement for ATM has not been demonstrated. The asterisk (*) beside serine 405 of TopBP1 indicates that phosphorylation of this residue is ATM-dependent, but phosphorylation is not required for localization of TopBP1 to foci following irradiation. The question mark following serine 20 of p53 reflects recent reports questioning the role of Chk2 in the phosphorylation of p53 at this site [107–109]. Although usually considered a substrate of ATR, Chk1 can be phosphorylated in an ATM-dependent manner on serines 317 and 345 [47,49], however, ATM-dependent phosphorylation of Chk1 at these sites is not required for its checkpoint function [49]. By mechanisms that are not yet understood, ATM is required for the Chk1-mediated phosphorylation and inactivation of Tousled-like kinases, Tlk1 and Tlk2 [110,111]. ATM is required for Chk2-mediated phosphorylation of BRCA1 on serine 988 [51], and phosphorylation of BRCA1 on serine 988 upregulates homologous recombination [50], thus, promoting DSB repair. ATM interacts with c-Abl [52], is required for IR-induced activation of c-Abl in vivo and phosphorylates c-Abl in vitro on serine 465, a site that is required for the ATM-dependent activation of c-Abl [48]. The question mark beside serine 465 of c-Abl reflects the fact

DNA-PKcs, or ATX, have similar substrate specificities and may be able to compensate for loss or dysfunction of ATM. However, the inter-relationship between the various PIKK family members remains to be clarified.

5. Monitoring DNA damage-induced activation of ATM—the problem with p53

Despite the abundance of physiological substrates of ATM, one of the most frequently used "read-outs" of ATM activity is the phosphorylation of p53 on serine 15. IR-induced phosphorylation of p53 is both attenuated and delayed in A-T cells [60]. However, serine 15 phosphorylation of p53 is clearly evident in ATM-deficient cell lines at later times after exposure to IR, indicating that ATM is required for serine 15 phosphorylation predominantly during the initial phase of the DNA damage response, and that other serine 15 specific protein kinases, such as ATR, can probably compensate for the absence of ATM at later times [60]. IR also induces phosphorylation of p53 at serines 6, 9, 20, 33, 46, 315, and 392, and ATM is required for efficient phosphorylation on serines 9, 20, and 46, as well as 15 [61]. Indeed, phosphorylation of p53 on serines 20 and 46 is far more dependent on the presence of ATM than is phosphorylation on serine 15 [61]. Therefore, in studying the requirement for ATM in the cellular response to a given stressor, the analysis of other ATM-dependent p53 phosphorylation sites, in particular serines 20 and 46, may be informative.

As shown in Fig. 2, although p53 is an important target of ATM, activation of ATM results in the phosphorylation of many other substrates and regulation of multiple cellular processes. Therefore, analysis of the ATM-dependent phosphorylation of one substrate cannot provide an accurate picture of the complexity of a given cellular response. A thorough understanding of the role of ATM will require a multifactorial approach in which the expression, activity, and phosphorylation of multiple substrates are analyzed both temporally and spatially. As discussed above, ATM is predominantly required for the initial response to DNA damage, therefore, time after damage must be an important experimental consideration when examining the potential role of ATM in a given response. Another important experimental consideration is the dose of a given DNA-damaging agent used, as alternative or redundant non-ATM-dependent pathways may be engaged at high levels of damage or cellular stress (see for example [31,36]).

6. Do other types of DNA damage trigger the activation of ATM?

To date, most studies have examined the effects of IR on the activation of ATM and ATM-dependent downstream pathways. IR induces damage to many biomolecules in the cell, including lipids and DNA (through the generation of single-strand breaks (SSBs), DSBs, and nucleotide damage), and triggers the formation of reactive oxygen species (ROS) (from ionization of water molecules and through lipid peroxidation). NCS, which also activates ATM, is a radiomimetic antibiotic that induces DSBs and SSBs by direct cleavage of the phosphodiester backbone [62]. ATM is also activated in response to DSBs induced during V(D)J recombination [63]. Together, these data support an important role for DSBs in the activation of ATM. Additional evidence comes from studies using bioflavonoids, many of which are topoisomerase (topo) II poisons. Genistein, a bioflavonoid that poisons topo II, induces phosphorylation of ATM on serine 1981, activation of the protein kinase activity of ATM, and ATM-dependent phosphorylation of p53 on serines 6, 9, 15, 20, 46, and 392 [64,65]. Other bioflavonoids that poison topo II [66] and activate ATM and/or ATM-dependent pathways include quercetin [65], kaempferol, apigenin, and luteolin [67]. Significantly, bioflavonoids such as biochanin A and daidzein, which are similar in structure to genistein but do not act as topo II poisons [66], do not induce phosphorylation of either ATM or p53 [65]. It is worth noting, however, that phosphorylation of ATM on serine 1981 does not necessarily indicate that downstream targets will be phosphorylated in an ATM-dependent manner. For example, although genistein, quercetin and etoposide are all topo II poisons and induce DSBs, only genistein-induced phosphorylation of p53 was dependent on the presence of ATM, whereas phosphorylation of p53 in response to either etoposide or quercetin was not [64,65].

7. Can ATM be activated in the absence of DNA damage?

Some studies have raised the interesting question of whether ATM can be activated by other forms of DNA damage, by changes in chromatin structure, or even by non-DNA-damaging events [12]. For example, serine 1981 phosphorylation of ATM occurs following exposure of cells to hypotonic buffer or to chloroquine, a topo II catalytic inhibitor, neither of which would be expected to induce DSBs [12]. Other studies have inferred activation of ATM and/or

Fig. 2. (*Continued*) that direct phosphorylation of this site has not been shown in vivo. c-Abl may play an important role in linking cell cycle checkpoint control to DSB repair by homologous recombination. ATM-activated c-Abl phosphorylates Rad51 on tyrosine 315 [53], and IR-induced Rad51 foci formation is defective in cells that lack either ATM or c-Abl [112]. c-Abl also phosphorylates BRCA1 in an ATM-dependent manner [54], while BRCA1 interacts with BRCA2 which, in turn, interacts with Rad51 (reviewed in [55]). For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.

Table 1
The effects of DNA-damaging and non-DNA-damaging agents on activation and phosphorylation of ATM and p53

Compound	DNA damage	ATM	p53
Radiation			
Ionizing radiation (^{137}Cs, ^{60}Co, X-rays)	ROS, SSB, DSB, base damage	Stimulation of kinase activity [20,22] Phosphorylation of serine 1981 [12]	ATM-dependent accumulation of p53 protein [60], ATM-dependent phosphorylation on serines 9, 15, 20, and 46 [61], Increased binding affinity to target sequence [60], Activation of downstream target genes (reviewed in [76])
Ultraviolet radiation (UVA)	ROS	Stimulation of kinase activity [77]	Accumulation of p53 protein [77], ATM-dependent phosphorylation on serines 15 and 20 [77]
Ultraviolet radiation (UVC)	Thymine dimers	No stimulation of kinase activity or phosphorylation of serine 1981 [12,20,22]	ATM-independent accumulation of p53 protein [22], ATM-independent phosphorylation on serines 9, 15, 20, and 46 [61]
Radiomimetics			
Neocarzinostatin	SSB, DSB	Stimulation of kinase activity [20] Phosphorylation of serine 1981 [31]	ATM-dependent accumulation of p53 protein [20], ATM-dependent phosphorylation on serine 15 [20]
Bleomycin	SSB, DSB	Phosphorylation of serine 1981[a,g]	ATM-dependent increase in p53 transcriptional activity [78]
Anti-tumor chemotherapeutics			
Etoposide	DSB[f]	Phosphorylation of serine 1981[b,h]	ATM-independent accumulation of p53 protein [64], ATM-independent phosphorylation on serine 15 [64]
Doxorubicin	DSB[f], ROS, DNA intercalator	Phosphorylation of serine 1981[c,i]	Accumulation of p53 protein [61,79,80], Phosphorylation on serines 6, 9, 15, 20, 33, 37, 46, 315, 392, and threonine 18 [61], ATM-dependent phosphorylation on serine 15 [80]
Camptothecin	SSB[e]	Phosphorylation of serine 1981[a,j]	Accumulation of p53 protein [81] Phosphorylation on serine 15 and serine 20 [81]
Cytosine arabinoside	Antimetabolite	ns[a,j]	Accumulation of p53 protein [82]
5-Fluorouracil	Antimetabolite	ns[a,j]	Accumulation of p53 protein [79]
Methotrexate	Antimetabolite	ns[a,k]	Accumulation of p53 protein [79]
Hydroxyurea	Ribonucleotide reductase inhibitor	ns[a,l]	ATM-independent accumulation of p53 protein [83], ATM-independent phosphorylation on serine 15 [83], Phosphorylation on serine 46 and serine 392 [83]
Alkylating/cross-linking agents			
N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)	Alkylating agent	Stimulation of kinase activity [84]	ATM-dependent phosphorylation on serine 15 [84]
Melphalan	Alkylating agent	ns[a,i,m]	nd
Carmustine (BCNU)	Alkylating agent	ns[a,g,n]	No accumulation of p53 protein [85]
Methyl methanesulfonate (MMS)	Alkylating agent	Low levels of serine 1981 phosphorylation[a,o]	Accumulation of p53 protein [85,86] Phosphorylation on serine 15 and serine 392 [85,86]
Mitomycin C	Cross-linking agent	ns[a,i]	Accumulation of p53 protein [79,85] Phosphorylation on serine 15 [85]
Cisplatin	Cross-linking agent	ns[a,j]	Accumulation of p53 protein [79,85] Phosphorylation on serine 15 and serine 20 [87]

Table 1 (*Continued*)

Compound	DNA damage	ATM	p53
Bioflavonoids			
Genistein	DSB[f], SSB[e], protein tyrosine kinase inhibitor	Stimulation of kinase activity [64] Phosphorylation of serine 1981 [65]	ATM-dependent accumulation of p53 protein [64], ATM-dependent phosphorylation on serines 6, 9, 15, 20, 46, and 392 [65], Increased binding affinity to target sequence [64]
Quercetin	DSB[f], protein tyrosine kinase inhibitor and serine/threonine kinase inhibitor	Stimulation of kinase activity [67] Phosphorylation of serine 1981 [65]	ATM-independent accumulation of p53 protein [65]. ATM-independent phosphorylation on serines 6, 9, 15, 20, 46, and 392 [65]
Luteolin	DSB[f], SSB[e]	Phosphorylation of serine 1981[a,k]	Accumulation of p53 protein [67] Phosphorylation on serine 15 [67]
Kaempferol	DSB[f], SSB[e]	Stimulation of kinase activity [67] Phosphorylation of serine 1981[a,k]	Accumulation of p53 protein [67] Phosphorylation on serine 15 [67]
Apigenin	DSB[f]	Stimulation of kinase activity [67] Phosphorylation of serine 1981[a,k]	Accumulation of p53 protein [67] Phosphorylation on serine 15 [67]
Biochanin A	None detected [66]	ns[a,k]	No accumulation or phosphorylation of p53 [65]
Daidzein	None detected [66]	ns[a,k]	No accumulation or phosphorylation of p53 [65]
Chromatin remodeling agents			
Chloroquine	Topo II catalytic inhibitor, DNA intercalator	Phosphorylation of serine 1981 [12]; ns[a,b,p]	Accumulation of p53 protein [12] Phosphorylation on serine 15 [12]
Sodium butyrate	Histone deacetylase inhibitor	ns[a,q]	Reduced levels of p53 protein [88] Increased phosphorylation of p53 [88]
Trichostatin A	Histone deacetylase inhibitor	Phosphorylation of serine 1981 at 10 μM [12]; ns[a,r]	nd
Ethidium bromide	Topo II catalytic inhibitor, DNA intercalator	ns[a,s]	No accumulation of p53 [79]
Actinomycin D	DNA intercalator	ns[a,t]	Accumulation of p53 protein [79,81,85], No phosphorylation on serine 15 and serine 20 [79,81,85]
Metabolic stresses			
Hypotonic buffer	?	Phosphorylation of serine 1981 [12]	Accumulation of p53 protein [12] Phosphorylation on serine 15 [12]
Reoxygenation after hypoxia	ROS-induced DNA damage?	nd	ATM-dependent phosphorylation on serine 15 [89]
Heat shock	ROS, other?	Stimulation of kinase activity [68]	ATM-dependent accumulation of p53 protein [68], ATM-dependent phosphorylation on serine 15 [68]
Glucose starvation	?	Stimulation of kinase activity [69]	Accumulation of p53 protein [69]
Other			
t-Butyl hydroperoxide	ROS	Stimulation of kinase activity [90]	ATM-dependent accumulation of p53 protein [90]
Hydrogen peroxide	ROS	Stimulation of kinase activity [91]	Accumulation of p53 protein [91] ATM-dependent phosphorylation on serine 15 [91]
Nitric oxide	ROS	nd	Accumulation of p53 protein [92] Phosphorylation on serine 15 partially ATM-dependent [92]

Table 1 (*Continued*)

Compound	DNA damage	ATM	p53
Okadaic acid	Protein phosphatase inhibitor	Phosphorylation of serine 1981[d]	Accumulation of p53 protein [93] Phosphorylation on serine 392 [93]
Sodium arsenite (arsenic(III))	ROS, DSB	nd	ATM-dependent accumulation of p53 protein [94], ATM-dependent phosphorylation on serine 15 [94], No phosphorylation on serine 15 [95]
Sodium chromate (chromium(VI))	DNA-cross-links	Stimulation of kinase activity [96]	ATM-dependent phosphorylation on serine 15 [96]
Single-stranded DNA	Genomic DNA strand breaks	ATM-dependent apoptosis [97]	Accumulation of p53 protein [97]
Tritiated nucleosides	?	nd	ATM-dependent accumulation of p53 protein [98]
Insulin	?	Stimulation of kinase activity [70]	nd

nd, not determined; ?, unknown; ns, not significant, defined as less than two-fold phosphorylation of serine 1981 over background levels under the conditions used. Superscripts g–s represent experiments carried out in a human lymphoblastoid cell line (BT) under the following conditions: g, 5 μM; h, 20 μM; i, 1 μM; j, 10 μM; k, 100 μM; l, 1 mM; m, 10 μM, 2 h; n, 5 or 50 μM, 48 h; o, 100 μg/ml; p, 32 μg/ml, 1 or 4 h; q, 5 or 0.5 mM, 24 h; r, 100 nM, 24 h; s, 50 μg/ml; t, 100 nM. Unless otherwise stated, the time of incubation with drug was 1 h.

[a] Kurz and Lees-Miller, unpublished data.

[b] Lees-Miller et al., unpublished data.

[c] Kurz and Lees-Miller, in preparation.

[d] Goodarzi and Lees-Miller, in preparation.

[e] Topo I poison.

[f] Topo II poison.

ATM-dependent downstream pathways from the induction of increased ATM activity in IP kinase assays and/or phosphorylation of p53 on serine 15 (Table 1). Although many of the agents used in these studies are known to induce DNA damage and, in many cases, DSBs, it is less clear how others, for example, heat shock [68], glucose starvation [69], or insulin treatment [70], induce DNA damage, thus, again suggesting that ATM may be activated in response to non-DNA damaging events. It is possible that certain cellular insults induce DSBs indirectly, through the inhibition of other cellular processes or by the induction of ROS. Indeed, many of the agents shown in Table 1 are known to produce ROS, and ROS play an important role in many aspects of ATM function, as well as the pathogenesis of A-T [71]. The availability of new tools (for example, phosphospecific antibodies to serine 1981 of ATM and other ATM substrates) and new models for activation of ATM (for example, the role of the MRN complex and BRCA1, Fig. 2), will allow the reported role of ATM in response to these agents to be assessed with more clarity.

8. Concluding remarks

If 1995 was a breakthrough year for identification of the *ATM* gene, then 2003 will stand out as the year in which seminal advances were made in understanding how ATM is activated in response to DNA damage, and perhaps other cellular stressors. However, certain key questions remain unanswered. Precisely how ATM and the MRN complex detect DNA damage and whether serine 1981 phosphorylation of ATM is required for the localization of ATM to sites of DNA damage remain to be determined. Precisely how changes in chromatin structure lead to activation of ATM [12], and how ATM is prevented from being activated during normal nuclear events, such as DNA replication and transcription, are also currently unclear. It is possible that histone modifications may signal the presence of DNA damage and/or changes in chromatin structure to ATM. Indeed, DNA damage-induced changes in histone H2AX phosphorylation have been well documented [34,72]. In addition, histone variant H1.2, a linker histone, was recently shown to be released from chromatin in response to DNA damage, inducing cytochrome *c* release and apoptosis [73,74]. Whether ATM plays a role in this process has not yet been determined, however, these studies provide another link between DNA damage, changes in chromatin structure, and downstream signaling events.

As shown in Table 1, many agents that are not known to induce DNA damage have been suggested to activate ATM; it will be interesting to determine whether these agents represent alternative mechanisms for ATM activation, or whether they, too, act directly or indirectly to induce DSBs and/or ROS. As discussed above, recent data suggest key roles for the MRN complex (possibly in complex with NFBD1/MDC1) and/or 53BP1 in IR-induced activation of ATM, and for BRCA1 in ATM-dependent downstream signaling events; whether MRN-NFBD1/MDC1, 53BP1 and BRCA1 are required for all ATM-dependent pathways in response to all DNA-damaging, and possibly

non-DNA-damaging, events remains to be determined. Also, one cannot ignore the apparent parallels between the potential role of the MRN complex in the activation of ATM and the role of the Ku70/80 dimer in the activation of the related protein, DNA-PKcs [75]. It will, therefore, be interesting to determine if the MRN complex stimulates the protein kinase activity of ATM in a similar manner. Also, it is almost certain that protein phosphatases will play important roles in regulating many aspects of ATM signaling, yet, at this time, very little is known about the role of protein phosphatases in regulating the DNA damage response. In conclusion, recent developments in our understanding of the mechanisms of ATM activation have provided us with important new tools for re-evaluating the role of ATM in signaling pathways and understanding the role of ATM in DNA damage and non-DNA damage responses, and answers to some of the questions raised here will likely be forthcoming in the near future.

Note added in proof

It has recently been shown that purified ATM and purified MRN complex physically interact and that MRN stimulates the protein kinase activity of ATM (J.H. Lee, T.T. Paull, Direct activation of the ATM protein kinase by the Mre11/Rad50/Nbs1 complex, Science 304 (2004) 93–96). Also, protein phosphatase 5 (PP5) interacts with ATM and regulates ATM-mediated DNA damage-induced signaling (A. Ali, J. Zhang, S. Bao, I. Liu, D. Otterness, N.M. Dean, R.T. Abraham, X.F. Wang, Requirement of protein phosphatase 5 in DNA-damage-induced ATM activation, Genes Dev. 18 (2004) 249–254).

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Appendix 6: Manuscript submitted to the Journal of Biological Chemistry

Doxorubicin activates ATM-dependent phosphorylation of multiple downstream targets in part through the generation of reactive oxygen species.

Ebba U. Kurz* and Susan P. Lees-Miller

Cancer Biology Research Group and Department of Biochemistry and Molecular Biology
University of Calgary, 3330 Hospital Drive NW, Calgary, AB, T2N 4N1 Canada

* To whom correspondence should be addressed:
Phone: (403) 220-7634
Fax: (403) 210-3899
Email: kurz@ucalgary.ca

Running title: Doxorubicin mediates ATM signalling through reactive oxygen species

The abbreviations used are: A-T, ataxia-telangiectasia; ATM, ataxia-telangiectasia mutated; DNA-PKcs, DNA-dependent protein kinase, catalytic subunit; DSB, DNA double-strand break; GSH, glutathione; Gy, Gray; IR, ionizing radiation; NAC, N-acetyl cysteine; PDTC, pyrrolidinedithiocarbamate; PIKK, phosphoinositide 3-kinase-like kinase; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; topo II, topoisomerase II.

SUMMARY

The requirement for the serine/threonine protein kinase ATM in co-ordinating the cellular response to DNA damage induced by ionizing radiation has been studied extensively. Many of the anti-tumour chemotherapeutics in clinical use today cause DNA double-strand breaks; however, few have been evaluated for their ability to modulate ATM-mediated pathways. We have investigated the requirement for ATM in the cellular response to doxorubicin, a topoisomerase II-stabilizing drug. Using several ATM-proficient and ATM-deficient cell lines, we have observed ATM-dependent nuclear accumulation of p53 and ATM-dependent phosphorylation of p53 on seven serine residues. This was accompanied by an increased binding of p53 to its cognate binding site, suggesting transcriptional competency of p53 to activate its downstream effectors. Treatment of cells with doxorubicin led to the ATM-dependent phosphorylation of histone H2AX on serine 139. Doxorubicin treatment also stimulated ATM autophosphorylation on serine 1981 and the ATM-dependent phosphorylation of numerous effectors in the ATM-signalling pathway, including Nbs1 (S343), SMC1 (S957), Chk1 (S317 and S345) and Chk2 (S33/35 and T68). Although generally classified as a topoisomerase II-stabilizing drug that induces DNA double-strand breaks, doxorubicin can intercalate DNA and generate reactive oxygen species. Pre-treatment of cells with the superoxide scavenger, ascorbic acid, had no effect on the doxorubicin-induced phosphorylation and accumulation of p53. In contrast, pre-incubation of cells with the hydroxyl radical scavenger, N-acetyl cysteine, significantly attenuated the doxorubicin-mediated phosphorylation and accumulation of p53, p53-DNA binding and the phosphorylation of Nbs1, SMC1, Chk1 and Chk2, suggesting that hydroxyl radicals contribute to the doxorubicin-induced activation of ATM-dependent pathways.

INTRODUCTION

DNA double-strand breaks (DSBs) are among the most cytotoxic DNA lesions. They arise through both endogenous (*e.g.* oxidative respiration) and exogenous (*e.g.* ionizing radiation (IR)) sources. In response to DSBs, cells must react immediately to repair the lesion, arrest the cell cycle to facilitate repair, or, in cases when damage is too extensive, initiate apoptosis.

Ataxia-telangiectasia mutated (ATM) is a member of the phosphoinositide 3-kinase-like family of serine/threonine protein kinases (PIKKs) (reviewed in (1-3)). ATM plays a central role in the cellular response to IR-induced DNA damage, essentially acting as a critical switch controlling if and when a cell arrests following DNA damage. In response to DNA DSBs induced by IR, ATM, which exists in an unstimulated cell as an inactive homodimer or higher order multimer, autophosphorylates to generate the active, monomeric kinase (4). Activation of ATM results in the phosphorylation of a diverse array of downstream targets that participate in numerous cellular events, including DNA damage recognition and processing, regulation of three cell cycle checkpoints (G_1, intra-S and G_2/M) and apoptosis (1-3). Among the most well-studied targets are the tumour suppressor protein p53 and the checkpoint kinase Chk2.

To date, most studies have investigated the effects of IR on the activation of ATM and ATM-dependent signalling pathways. IR is a potent DNA-damaging agent, inducing both DNA single-strand breaks and DSBs, in large part through the actions of reactive oxygen species (ROS) generated by the ionization of water molecules in the cell and through lipid peroxidation. In addition to IR, many of the anti-tumour chemotherapeutics commonly used in the treatment of cancer induce, either directly or indirectly, DSBs, yet, at present, few DNA-damaging chemotherapeutics have been evaluated for their ability to activate ATM and ATM-dependent signalling pathways. It is well established, however, that numerous anti-cancer drugs induce the

nuclear accumulation of p53 (5;6). The ability of these chemotherapeutics to induce p53 accumulation has been correlated directly with the DNA damaging capacity of the drug (5).

Several key pieces of evidence support a role for ATM in drug-induced DNA damage. Recently, arsenite, a potent human carcinogen that induces DSBs, was reported to induce p53 accumulation in an ATM-dependent manner (7). This increase in p53 was linearly correlated with strand break induction. Hexavalent chromium (Cr(VI)), a broad-spectrum DNA-damaging agent, activates ATM kinase activity and induces the phosphorylation of p53 on serine 15 (8). Genistein, a tyrosine kinase inhibitor and topoisomerase II (topo II) poison, activates ATM protein kinase activity, induces phosphorylation of ATM on serine 1981 and the ATM-dependent phosphorylation of histone H2AX on serine 139 and p53 on multiple serine residues (9;10). Although not classically considered a DNA-damaging chemotherapeutic, the monofunctional DNA alkylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) stimulates ATM kinase activity and the ATM-dependent phosphorylation of p53 on serine 15, possibly triggered by the strand breaks created during the DNA repair process (11). Given the critical role for ATM in the cellular response to DSBs and the prominent, though not exclusive, role for ATM in the phosphorylation of p53 in response to DNA damage, we sought to examine the effects of the anti-tumour anthracycline, doxorubicin, on ATM and its downstream effectors.

EXPERIMENTAL PROCEDURES

Reagents: Doxorubicin, wortmannin, ascorbic acid and N-acetyl cysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of doxorubicin and wortmannin were prepared in dimethylsulfoxide, protected from light and stored at -20°C. Stock solutions of ascorbic acid and NAC were prepared fresh in 0.9% NaCl, with the pH of the NAC stock solution adjusted to pH 7.5.

Cells: ATM-proficient (BT and C3ABR) and ATM-deficient (L3 and AT1ABR) human lymphoblastoid cell lines were as previously described (9;10). Cells were maintained as suspension cultures in either RPMI 1640 (BT and L3) or DMEM/F12 (C3ABR and AT1ABR) media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 50 U/ml penicillin G and 50 μg/ml streptomycin sulfate. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Chemotherapeutics, inhibitors, antioxidants or equivalent volumes of carrier were added directly to the cell media at the start of each experiment, unless otherwise stated. Where indicated, cells were irradiated in the presence of serum-containing medium using a Gammacell 1000 ^{137}Cesium source (MDS Nordion, Ottawa, Canada).

Antibodies: The p53-specific monoclonal antibody DO-1, agarose-conjugated DO-1, agarose-conjugated Pab1801, and an antibody specific for histone H2A (H-124) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphospecific antisera to serines 6, 9, 15, 20, 37, 46 and 392 of human p53, serines 317 and 345 of Chk1, serines 33/35 and threonine 68 of Chk2 were purchased from Cell Signaling Technology (New England Biolabs, Beverly, MA), as was

an antibody reactive for total pool of Chk1. Phosphospecific antisera to serine 343 of Nbs1 and serine 957 of SMC1 were purchased from Novus Biologicals (Littleton, CO), as were antisera reactive to the total pools of Chk2, Nbs1 and SMC1. A polyclonal antibody to actin (A2066) was acquired from Sigma-Aldrich. A phosphospecific antiserum to serine 1981 of human ATM was purchased from Rockland Immunochemicals (Gilbertsville, PA). A phosphospecific antibody to serine 139 of human histone H2AX was purchased from Upstate USA, Inc (Lake Placid, NY). A rabbit polyclonal antibody specific for human ATM (4BA) was a generous gift from Dr. Martin Lavin (Queensland Institute for Medical Research). DPK1, a polyclonal antibody specific for the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) was raised against a recombinant protein fragment (amino acids 2018-2136) and has been previously described (12).

Immunoblots and Immunoprecipitation: Crude nuclear protein extracts (500 mM NaCl extraction) were prepared from logarithmically growing cells, as previously described (9). Protein concentrations were determined using the Bradford-based Bio-Rad protein assay (Bio-Rad, Hercules, CA) using bovine serum albumin as the standard. For immunoblots, 30 μg of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and probed with antibodies to p53 (DO-1), actin or a phosphospecific antiserum to p53 phosphorylated at serine 15.

Detection and analysis of p53 phosphorylation at serines 6, 9, 20, 37, 46 and/or 392 were performed after immunoprecipitation of p53 using agarose-conjugated DO-1 and Pab1801 antibodies as described previously (13). Immunoprecipitation/immunoblot experiments for the detection of p53 phosphorylation at these sites were carried out as described (10).

For the analysis of ATM phosphorylation at serine 1981 and the phosphorylation of other downstream effectors of ATM, whole cell extracts were prepared from logarithmically growing cells. Briefly, 8-10 x 10^6 cells were harvested, washed twice in phosphate-buffered saline and lysed by sonication in NET-N buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0, 1% (v/v) Nonidet P-40) containing protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A) and phosphatase inhibitors (1 mM activated Na_3VO_4, 25 mM NaF, 1 μM microcystin-LR). Protein concentrations of cleared lysates were determined using a Lowry-based, detergent-compatible protein assay (Bio-Rad) using bovine serum albumin as standard. For immunoblots examining ATM, DNA-PK or SMC1, 60 μg of protein were separated on 8% SDS-PAGE gels (30:0.25 acrylamide:bis-acrylamide) and transferred to nitrocellulose in SDS-electroblot buffer (25 mM Tris, 192 mM glycine, 0.04% (w/v) SDS, 20% (v/v) methanol) at 100V for 60 min. For all other proteins, 60 μg of protein were separated on 10% SDS-PAGE gels (29.2:0.8 acrylamide:bis-acrylamide) and transferred to nitrocellulose as described above, but without the addition of SDS to the electroblot buffer.

Electrophoretic Mobility Shift Assay: Electrophoretic mobility shift assays using crude nuclear extracts (500 mM NaCl extraction) were performed as described previously (9).

Isolation and Analysis of Histones: For the detection of histone H2AX, cells were harvested, washed and lysed in ice-cold buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, pH 8.0, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1% (v/v) Triton X-100) containing protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride,

1 μg/ml pepstatin A) and phosphatase inhibitors (1 mM activated Na_3VO_4, 25 mM NaF, 1 μM microcystin-LR) and sonicated. Protein concentrations of cleared lysates were determined using a Lowry-based, detergent-compatible protein assay (Bio-Rad) using bovine serum albumin as standard. Sixty μg of protein were analyzed by SDS-PAGE on 15% polyacrylamide gels (29.2:0.8 acrylamide:bis-acrylamide). Proteins were transferred onto a nitrocellulose membrane at 100V for 60 min in a buffer containing 48 mM Tris base, 39 mM glycine, 0.04% (w/v) SDS and 10% methanol. Immunoblots were probed first for phosphorylation of histone H2AX at serine 139. Membranes were then stripped and reprobed with an antibody to total histone H2A.

Image Analysis: Image analysis was performed using ImageQuant software (Molecular Dynamics, Amersham Pharmacia Biotech). In the evaluation of specific phosphorylation events, phosphorylation levels were normalized to total protein levels by dividing the intensity of the phosphospecific signal by the intensity of the signal from measured from blots using antibodies recognizing the total pool of protein.

RESULTS

Doxorubicin induces ATM-dependent stabilization and phosphorylation of p53 on serine 15:

Previous studies have shown that phosphorylation of p53 in response to IR is mediated by the ATM protein kinase (14;15) and that ATM is important for p53 stabilization and for stimulating the *trans*-activation functions of p53 at early times after IR. Doxorubicin has previously been shown to stimulate the nuclear accumulation and phosphorylation of p53 (5;13), but neither of these studies investigated the requirement for ATM in these events. Here, we demonstrate that doxorubicin-induced stabilization and phosphorylation of p53 on serine 15 at early time points following treatment occur only in the presence of the ATM protein kinase (Fig. 1A and B). Treatment of ATM-proficient BT cells with doxorubicin (1 μM) induced phosphorylation of p53 on serine 15 within 60 min, further increasing at 120 min to a level comparable to that observed with exposure to IR (10 Gy, 2hr). The phosphorylation was absent in the ATM-deficient L3 cells at the times examined, however, in a manner similar to IR (16), doxorubicin induced a modest accumulation of p53 and phosphorylation at serine 15 in ATM-deficient cells at later time points (4 hr and longer) (data not shown). Similar results were obtained in experiments with a second pair of ATM-proficient (C3ABR) and ATM-deficient (AT1ABR) cell lines (data not shown).

To confirm further the role of ATM in the phosphorylation and accumulation of p53 in response to doxorubicin, cells were pretreated with wortmannin, a fungal metabolite that binds irreversibly to the ATP binding site of PIKKs (17;18). ATM-proficient BT cells were pretreated for thirty minutes with increasing concentrations of wortmannin prior to the addition of doxorubicin and incubations were continued for a further two hours. As shown in Fig. 1C, pretreatment of cells with 10 μM wortmannin reduced both the accumulation and serine 15

phosphorylation of p53 to basal levels. Similar results were observed with a second ATM-proficient human lymphoblastoid cell line (C3ABR, data not shown).

Doxorubicin induces ATM-dependent phosphorylation of p53 on serines 6, 9, 15, 20, 37, 46 and 392:

In response to IR, p53 becomes phosphorylated on at least eight serine residues (located at amino acids 6, 9, 15, 20, 33, 46, 315 and 392), and it has been demonstrated that ATM is required for the early phosphorylation of the amino-terminal serines at positions 9, 15, 20 and 46, with only weak phosphorylation evident in ATM-deficient cells at later time points (13). To evaluate whether p53 is also phosphorylated at multiple serine residues in response to doxorubicin, and the ATM-dependence of any such post-translational modifications, p53 was immunoprecipitated from doxorubicin-treated ATM-proficient (BT) and ATM-deficient (L3) cells and immunoblotted with phosphospecific antisera to p53. Doxorubicin treatment induced the phosphorylation of p53 at serines 6, 9, 20, 37, 46 and 392, and, in all cases, phosphorylation was ATM-dependent (Fig. 2). In contrast, only very weak, if any, phosphorylation was observed at a later time point (4 hr) in ATM-deficient cells. Very similar results were obtained in C3ABR (ATM-proficient) and AT1ABR (ATM-deficient) cells (data not shown).

Doxorubicin stimulates p53-DNA binding in ATM-competent cells:

To determine whether the doxorubicin also stimulated p53 to bind its cognate DNA binding site, nuclear extracts isolated from doxorubicin-treated ATM-proficient (BT) and ATM-deficient (L3) cells were analyzed by electrophoretic mobility shift assay. Treatment of ATM-proficient cells with doxorubicin was found to increase dramatically the ability of p53 to bind its

cognate DNA-binding site (Fig. 3). In contrast, binding of p53 to DNA was significantly reduced in ATM-deficient cells.

Doxorubicin induces phosphorylation of ATM on serine 1981:

Stimulation of ATM kinase activity following irradiation has recently been demonstrated to occur after autophosphorylation of ATM on serine 1981 (4). To examine whether doxorubicin could also induce autophosphorylation of ATM on serine 1981, ATM-proficient (BT) and ATM-deficient (L3) cells were treated with doxorubicin (1 μM) for two hours prior to extract preparation and immunoblotting with a phosphospecific antiserum to serine 1981 of ATM. Exposure to doxorubicin induced marked phosphorylation of ATM at serine 1981, to a level comparable to that induced by exposure to IR (10 Gy, 2 hr) (Fig. 4A).

Doxorubicin induces ATM-dependent phosphorylation of multiple downstream effectors in the ATM signalling pathway:

Although p53 is an important target of ATM, activation of ATM results in the phosphorylation of a diverse array of downstream targets that participate in multiple cellular processes. Analysis of ATM-dependent phosphorylation of one substrate cannot provide an accurate picture of the complexity of the cellular response. To gain a broader perspective on the requirement for ATM in the early cellular response to doxorubicin treatment, ATM-proficient (BT) and ATM-deficient (L3) cells were treated with doxorubicin (1 μM) and incubated for 60 min or 120 min prior to extract preparation and immunoblotting with phosphospecific antisera to known downstream effectors of ATM. Exposure to doxorubicin induced the ATM-dependent phosphorylation of all substrates tested. Interestingly, the substrates appear to stratify into two

groups, early phosphorylation events (≤60 min) and late phosphorylation events (>60 min). Serine 15 of p53 (Fig. 1A), threonine 68 of Chk2 (Fig. 4B) and serine 343 of Nbs1 (Fig. 4B) were all phosphorylated in an ATM-dependent manner within sixty minutes of the initiation of doxorubicin treatment. In contrast, ATM-dependent phosphorylation of Chk1 on serines 317 and 345, Chk2 on serines 33/35 and SMC1 on serine 957 were only detectable two hours after initiating doxorubicin treatment (Fig. 4B). Interestingly, ATM-dependent phosphorylation of Chk1 at serines 317 and 345 in response to either doxorubicin or IR was also accompanied by increased immunoreactivity with the antibody for the total cellular pool of Chk1 (Chk1, Fig. 4B). Whether this, in a manner similar to p53, represents protein stabilization and accumulation, or whether the phosphorylated protein takes on a secondary structure with higher affinity for the antiserum remains to be determined.

Within its amino-terminal domain, Chk2 contains a cluster of seven potential ATM phosphorylation sites, of which threonine 68 has been identified as the major *in vivo* ATM phosphorylation site (19-21). While threonine 68 of Chk2 is the primary site of ATM-directed phosphorylation, other sites within this cluster are phosphorylated to a lesser extent, and the amino-terminal 57 amino acids are required, at least *in vitro*, for the efficient phosphorylation of Chk2 by ATM (22). Phosphorylation of Chk2 is accompanied by a reduction in the electrophoretic mobility of Chk2. In response to treatment with doxorubicin, phosphorylation of Chk2 at threonine 68 is readily detectable within sixty minutes and precedes the appearance of an electrophoretically retarded, hyperphosphorylated species of Chk2 (Fig. 4B), suggesting that threonine 68 is one of the first residues in Chk2 to be phosphorylated following exposure to doxorubicin. This is in contrast to the phosphorylation of Chk2 at either or both serine 33 and serine 35 that is detectable only after 120 minutes and only in the hyperphosphorylated form of

Chk2 with reduced electrophoretic mobility (Fig. 4B). In an inverse manner to that of Chk1, the appearance of this hyperphosphorylated form of Chk2 is accompanied by reduced immunoreactivity with the antibody for the total cellular pool of Chk2 (Chk2, Fig. 4B). Given the increased abundance of the phosphorylated forms of the protein, this is unlikely to represent protein destabilization, but rather may reflect a change in the secondary structure with reduced affinity for the antiserum.

In a manner similar to Chk2, Nbs1 is phosphorylated in an ATM-dependent manner at multiple serine residues, including serine 343 (23-26). This is accompanied by a reduction in the electrophoretic mobility of Nbs1. In response to treatment with doxorubicin, phosphorylation of Nbs1 at serine 343 is detectable within sixty minutes of treatment and precedes the appearance of a reduced mobility form of Nbs1 (Fig. 4B), suggesting that serine 343 is one of the first residues in Nbs1 to be phosphorylated following treatment with doxorubicin.

Doxorubicin induces ATM-dependent phosphorylation of histone H2AX:

A very early and sensitive marker of DSB induction is the phosphorylation of histone H2AX on serine 139 (27;28). In response to IR, this phosphorylation event has been shown to be mediated in a redundant manner by ATM and DNA-PKcs, however, in lymphoblastoid cell lines, H2AX phosphorylation appears to be predominantly ATM-dependent (29). In keeping with this observation, we have previously reported, using the BT (ATM-proficient) and L3 (ATM-deficient) human lymphoblastoid cell lines, that H2AX phosphorylation in response to IR is ATM-dependent (10). To determine if doxorubicin induces phosphorylation of histone H2AX and the requirement of ATM in this process, extracts were prepared from logarithmically growing, doxorubicin-treated BT and L3 cells and probed for phosphorylation of histone H2AX

at serine 139. Doxorubicin-induced phosphorylation of histone H2AX at serine 139 was observed within sixty minutes of doxorubicin treatment, increasing further at 120 minutes in the ATM-proficient BT cells, but not in the ATM-deficient L3 cells (Fig. 5). Similar results were observed with doxorubicin treatment in C3ABR (ATM-proficient) and AT1ABR (ATM-deficient) cells.

N-acetyl cysteine abrogates doxorubicin-mediated stabilization and phosphorylation of p53 and attenuates p53-DNA binding:

Although generally classified as a topo II-stabilizing drug that induces DSBs, doxorubicin can intercalate DNA and generate ROS through the reaction of its quinone moiety with cytochrome P450 reductase and NAD(P)H (30). To evaluate a possible role for ROS in doxorubicin-mediated effects on p53, ATM-proficient BT cells were pretreated with antioxidants prior to treatment with doxorubicin. Pretreatment of cells with the superoxide scavenger (31) ascorbic acid had no effect on the doxorubicin-induced stabilization and phosphorylation of p53 on serine 15 (Fig. 6A). In contrast, preincubation of cells with the hydroxyl radical scavenger (31;32) NAC significantly attenuated the doxorubicin-mediated stabilization and phosphorylation of p53 on serine 15 (Fig. 6B), suggesting that hydroxyl radicals may play a role in doxorubicin-induced activation of ATM-dependent pathways. Consistent with the reduced phosphorylation and accumulation of p53 in doxorubicin-treated cells pretreated with NAC, p53 in extracts prepared from these cells showed a dramatically reduced ability to bind its cognate DNA-binding site (Fig 6C). Similar results were observed with antioxidant pretreatment in C3ABR (ATM-proficient) cells.

Pretreatment with N-acetyl cysteine attenuates the doxorubicin-mediated, ATM-dependent phosphorylation of multiple downstream effectors in the ATM signalling pathway:

To gain a broader perspective on the role of hydroxyl radicals in the early cellular response to doxorubicin treatment, ATM-proficient BT cells were pre-treated for thirty minutes with NAC prior to the addition of doxorubicin (1 μM). The incubation was continued for a further 60 min or 120 min, extracts were then prepared and immunoblotted with phosphospecific antisera to known downstream effectors of ATM. Pretreatment of cells with NAC significantly attenuated or delayed the doxorubicin-induced phosphorylation of all substrates tested (Fig. 7A and B). For ATM, Nbs1 and SMC1, pretreatment with NAC only partially attenuated the observed phosphorylation, whereas for Chk1 and Chk2, NAC pretreatment led to a near complete abrogation of the doxorubicin-induced phosphorylation. Similar results were observed with NAC pretreatment in the ATM-proficient C3ABR cell line.

Qualitatively similar results were observed in cells pretreated with pyrrolidinedithiocarbamate (PDTC), another hydroxyl radical scavenging antioxidant. In addition to its antioxidant properties, paradoxically, PDTC can also function as an oxidant and in cells treated with PDTC alone ATM, Nbs1, Chk1 (serine 345) and Chk2 (threonine 68) phosphorylation was observed (data not shown). Interestingly, subsequent incubation with doxorubicin did not induce further phosphorylation of ATM or Chk2, nor was SMC1 phosphorylation detectable with sequential incubations of PDTC and doxorubicin (data not shown).

DISCUSSION

The serine/threonine protein kinase, ATM, plays a critical role in the cellular response to DNA damage. Exposure to IR generates DSBs leading to the rapid activation of ATM in the cell. Interestingly, many of the anticancer drugs in active clinical use today also have the capacity to induce DSBs, however, little is known about the role of ATM in response to the damage induced by these drugs. We present here that doxorubicin, a topo II poison, induces ATM autophosphorylation and the ATM-dependent phosphorylation of multiple downstream effectors within the DNA damage response pathway. We further present evidence that ROS, specifically hydroxyl radicals, participate in the doxorubicin-mediated activation of this complex pathway.

A previous study has shown that p53 is phosphorylated at four serine residues in an ATM-dependent manner in response to IR (13). In contrast, doxorubicin induced the phosphorylation of p53 at serines 6, 9, 15, 20, 37, 46, 392, and, in all cases, phosphorylation was ATM-dependent. In response to genistein, a plant isoflavonoid, p53 is phosphorylated at six serine residues (6, 9, 15, 20, 46 and 392) in an ATM-dependent manner, while the related bioflavonoid quercetin induced phosphorylation at these sites in a strictly ATM-independent manner (10). It is becoming clear that multi-site phosphorylation is a dynamic and powerful method of delicately modulating the activity of proteins within the cell. Phosphorylation at different regions within a cell can control localization, stability, protein-protein interaction, DNA-binding activity and enzymatic activity, among others (33). In the case of p53, initial studies demonstrated that casein kinase-1-dependent phosphorylation of threonine 18 is dependent on the prior phosphorylation of serine 15 (34;35). In addition, acetylation of p53 at lysines 320 and 383 requires the prior phosphorylation of p53 at serine 15, and the

phosphorylation of additional amino-terminal sites further stimulates these acetylation events (13). Recent reports have presented evidence for much more extensive interdependence in the phosphorylation of amino-terminal residues in p53 (36). Prior phosphorylation of serine 15 appears to be required for the efficient phosphorylation of serine 9, serine 20 and threonine 18, whereas serines 6 and 9 are dependent upon one another for phosphorylation without affecting the phosphorylation of other residues in the amino-terminus of p53 (36). Clearly, the phosphorylation of p53 is regulated in an intricate and dynamic manner. The role of ATM is this process is equally complex, responding to a specific subset of chemotherapeutics and DNA-damaging agents, each triggering a unique pattern of downstream post-translational modifications.

Although primarily regarded as a topo II poison, numerous cellular effects of doxorubicin are mediated through its generation of ROS. Recently, it has been demonstrated that prolonged treatment of cells with doxorubicin (0.86 μM, 24-120hr) leads to an increase in p53 protein levels, followed by the p53-mediated transcriptional upregulation of manganese superoxide dismutase and glutathione peroxidase-1 (37). This was associated with an increased production of ROS and co-treatment with NAC was shown to significantly reduce the number of apoptotic cells. Through the use of chemical antioxidants, we have shown that hydroxyl radicals play a role in the doxorubicin-induced activation of ATM-dependent pathways. Whether the cellular response to doxorubicin treatment is a direct effect of the ROS, or secondary to DSBs generated by their reaction with DNA remains unclear. It is tempting to speculate that the partial suppression of ATM, Nbs1 and SMC1 phosphorylation by NAC pretreatment (Fig. 7) reflects the dualistic nature of doxorubicin; that some level of phosphorylation is attributable to the ROS generated by doxorubicin, while the balance reflects the DSBs generated by doxorubicin through

its stabilization of topo II-DNA cleavable complexes. Were this the case, it would suggest that the phosphorylation of Chk1 and Chk2 reflect an oxidative stress response more than a direct DNA damage response.

In the cell, ATM is predominantly required for the early (minutes to hours) response to DNA damage, while other PIKKs, such as ATR, can complement the response at later time points or, in the case of cells lacking ATM, compensate for its absence. Therefore, time after damage must be an important experimental consideration when studying the role for ATM in any given response (3;38). Keeping this in mind, all experiments presented herein were conducted within four hours of cell treatment. Several previous studies have assessed the role of ATM in the cellular response to doxorubicin. Some of these used very late time points (16-24 hours) and, hence, interpretation of the data may be hampered by the activation of redundant pathways (39-41). Other studies have examined early time points (up to four hours) and have demonstrated that doxorubicin induces the ATM-dependent phosphorylation of p53 at serine 15 (42) and activates a MEK/ERK pathway leading to stimulation of IκB kinase activity and activation of the pro-survival transcription factor NF-κB in an ATM-dependent manner (43). Interestingly, it has been well-studied that the expression and function of NF-κB are upregulated in response to ROS (44), although the role for ROS in the doxorubicin-induced activation of NF-κB remains to be studied.

Inherited defects in the gene coding for ATM lead to development of ataxia-telangiectasia (A-T). Consistent with ATM's central role in cell cycle regulation in response to DNA damage, this autosomal recessive disorder is characterized by profound sensitivity to IR, cancer predisposition, immunodeficiency, genomic instability and a progressive loss of motor control due to cerebellar ataxia (reviewed in (2;45)). A multitude of studies have supported a

role for ROS in aspects of ATM function, as well as the pathogenesis of A-T (reviewed in (46-48)). It has been suggested that ATM could be a sensor of perturbations in redox homeostasis or oxidative damage, triggering the activation of signal transduction pathways responsible for protecting cells from such insults (46;48). Thus, the absence of functional ATM would result in cells under a continuous state of oxidative stress. Consistent with this are observations that A-T cells and tissues exhibit significantly reduced rates of glutathione (GSH) re-synthesis following depletion (49), show reduced levels of nicotine adenine dinucleotide (50) and elevated levels of numerous biomarkers of oxidative damage (51). We demonstrate here that hydroxyl radicals play a role in the rapid activation of ATM and ATM-dependent signalling pathways which further supports the hypothesized link between ATM function and ROS. Interestingly, doxorubicin has been demonstrated in mice to induce an immediate and acute reduction in GSH levels in erythrocytes, liver and cardiac tissue, and the administration of thiol donors (cysteamine or NAC) prevents this fall (52). It is tempting to speculate, given this and the impaired recovery from GSH depletion in A-T cells (49), that ATM may play a role, either directly or indirectly, in modulating the GSH biosynthesis/recycling pathway.

Although A-T is rare, studies suggest that 1-2% of the general population is heterozygous for mutations in *ATM* and clinical and epidemiological evidence points to an increased cancer risk, particularly breast cancer, within this carrier population (53). In addition, these carriers have an intermediate sensitivity to IR (54;55). Interestingly, many of the anti-tumour chemotherapeutics used in the treatment of breast cancer have the capacity to induce DSBs or generate ROS. For breast cancer patients heterozygous for mutations in *ATM*, exposure to these drugs or IR could lead to more profound manifestations of side effects or the increased incidence of secondary, treatment-related malignancies. Identification of drugs that do not activate ATM

could lead to modified treatment protocols for these patients with the aim of reducing side effects and improving the long-term outcome of therapy.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Doxorubicin-induced accumulation of p53 and p53 phosphorylation on serine 15 require ATM and are abrogated by pretreatment with wortmannin. *Panel A:* ATM-proficient (BT) and ATM-deficient (L3) human lymphoblastoid cells were treated with doxorubicin (1 μM) and harvested at the indicated times. Nuclear extracts were prepared and analyzed by sequential immunoblotting using a phosphospecific antiserum to serine 15 of p53 (p53 pS15), a pan-specific antibody (DO-1) to p53 (p53) and a polyclonal antiserum to actin. An extract from BT cells irradiated with 10 Gy IR and allowed to recover for two hours served as a positive control (IR). *Panel B:* The immunoblots shown in Panel A were scanned, quantitated and serine 15 phosphorylation was normalized to total levels of p53 (as judged by immunoreactivity with the DO-1 antibody and described in Experimental Procedures). Hatched bars represent the ATM-proficient BT cells, solid bars represent the ATM-deficient L3 cells. *Panel C:* ATM-proficient cells (BT) were either pre-treated with DMSO (0 μM wortmannin, lanes 1 and 2) or increasing concentrations of wortmannin (lanes 3-5) for 30 min prior to the addition of 1 μM doxorubicin (lanes 2-5) and further incubation for two hours. Nuclear extracts were prepared and analyzed by immunoblotting as for Panel A.

Figure 2. Doxorubicin induces ATM-dependent phosphorylation of p53 on serines 6, 9, 20, 37, 46 and 392. *Panel A:* ATM-proficient (BT) and ATM-deficient (L3) cells were treated with doxorubicin (1 μM) and harvested at the indicated times. To determine the phosphorylation state of p53 at the indicated serines, p53 was immunoprecipitated from whole cell extracts and analyzed by immunoblotting, as described in the Experimental Procedures, using phosphospecific antisera, followed by incubation with the monoclonal antibody DO-1 for total

p53. *Panel B:* The immunoblots shown in Panel A were scanned, quantitated and p53 phosphorylation at individual sites was normalized to total p53 (as judged by immunoreactivity with the DO-1 antibody). Hatched bars represent the ATM-proficient BT cells, solid bars represent the ATM-deficient L3 cells. In the case of phosphorylation of p53 at serine 37, an elevated, uneven background on the immunoblot precluded an accurate quantitative assessment of p53 phosphorylation at this residue in the ATM-deficient L3 cells. Hence, data for this are not included in the graph.

Figure 3. Doxorubicin stimulates p53-DNA binding in ATM-proficient (BT) cells. Oligonucleotides containing a consensus p53 binding site were annealed and end-labelled with [γ-^{32}P]ATP. Nuclear extracts (9 μg of protein) from untreated or doxorubicin-treated (1 μM, 2 hours) ATM-proficient (BT) or ATM-deficient (L3) cells were assayed for binding activity to the ^{32}P-labeled binding site in the presence of 1 μg poly(dI-dC)·poly(dI-dC) and 4 μl of the p53 monoclonal antibody Pab421 (to stabilize the binding of p53 to its cognate binding site (56)). The DNA-protein complex (bound) was separated from free probe (free) by electrophoresis through a non-denaturing, 4.5% polyacrylamide gel.

Figure 4. Doxorubicin induces the autophosphorylation of ATM on serine 1981 and ATM-dependent phosphorylation of multiple downstream effectors in the ATM signalling pathway. *Panel A:* ATM-proficient (BT) and ATM-deficient (L3) cells were treated with doxorubicin (1 μM, 2 hours) or exposed to 10 Gy IR and allowed to recover for two hours prior to harvest. Whole cell extracts were prepared and analyzed by sequential immunoblotting using a phosphospecific antiserum to serine 1981 of ATM, a pan-specific antiserum to ATM (4BA) and

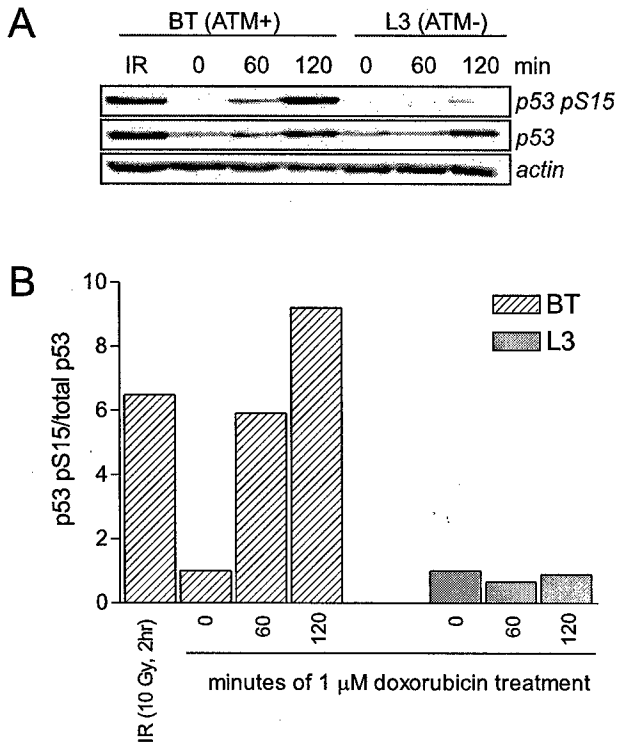
a polyclonal antiserum to DNA-PKcs (to verify the loading of comparable protein levels in all lanes). *Panel B:* ATM-proficient (BT) and ATM-deficient (L3) cells were treated with doxorubicin (1 μM) and harvested at the indicated times. Whole cell extracts were prepared and the phosphorylation status of downstream effectors within the ATM signalling network was analyzed by immunoblotting with available phosphospecific antisera. An extract from BT cells irradiated with 10 Gy IR and allowed to recover for two hours served as a positive control (IR).

Figure 5. Doxorubicin induces the ATM-dependent phosphorylation of histone H2AX on serine 139. *Panel A:* ATM-proficient (BT) and ATM-deficient (L3) cells were treated with doxorubicin (1 μM) and harvested after 30, 60 or 120 min, as indicated. Histones were extracted as described in Experimental Procedures, and 40 μg of protein were analyzed on 15% SDS-PAGE gels, transferred to nitrocellulose and probed with a phosphospecific antibody to serine 139 of histone H2AX (γH2AX pS139). The immunoblot was then stripped and probed for histone H2A. An extract from BT cells irradiated with 10 Gy IR and allowed to recover for 30 min served as a positive control (IR) (10). *Panel B:* The immunoblots shown in Panel A were scanned, quantitated and serine 139 phosphorylation was normalized to total levels of histone H2A. Data for each cell line were then expressed as the fold-induction of phosphorylated γH2AX over the untreated control. Hatched bars represent the ATM-proficient BT cells, solid bars represent the ATM-deficient L3 cells.

Figure 6. NAC attenuates the doxorubicin-mediated induction of p53 phosphorylation and accumulation and p53-DNA binding. *Panels A and B:* ATM-proficient (BT) cells were either pre-treated with 0.9% NaCl (0, lanes 1 and 3) or increasing concentrations of ascorbic acid

(Panel A, lanes 2 and 4-6) or NAC (Panel B, lanes 2 and 46) for 30 min prior to the addition of 1 μM doxorubicin (lanes 3-6) and further incubation for 2 hours. Nuclear extracts were prepared and analyzed by immunoblotting as for Figure 1. *Panel C:* Electrophoretic mobility shift assays were carried out with the extracts from lanes 1, 3 and 6 in Panel B as described for Figure 3.

Figure 7. The hydroxyl radical scavenger, NAC, attenuates the doxorubicin-mediated, ATM-dependent phosphorylation of multiple downstream effectors in the ATM-signalling pathway. *Panel A:* ATM-proficient (BT) cells were either pre-treated with 0.9% NaCl (lanes 1-3) or 50 mM NAC (lanes 4-6) for 30 min prior to the addition of 1 μM doxorubicin and further incubation for 60 or 120 min. Whole cell extracts were prepared and the effect of antioxidant pretreatment on the phosphorylation status of downstream effectors within the ATM signalling network was analyzed by immunoblotting with phosphospecific antisera. *Panel B:* The immunoblots shown in Panel A were scanned, quantitated and phosphorylation at each site was normalized to total levels of each respective protein analyzed. To account for any basal effect of the pretreatment alone, data for each condition (doxorubicin alone or NAC pretreatment) were then expressed as the fold-induction of phosphorylation over the doxorubicin-untreated control within each set (lanes 1 and 4). Hatched bars represent treatment with doxorubicin alone (no pretreatment) and cross-hatched bars represent pretreatment with NAC (50 mM, 30 min).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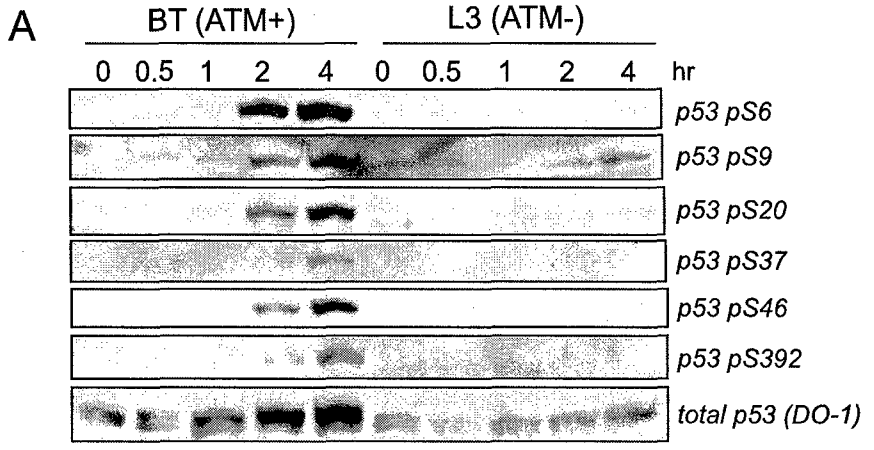
BT (ATM+)
L3 (ATM-)
IR 0 60 120 0 60 120 min
p53 pS15
p53
actin
B
p53 pS15/total p53
BT
L3
IR (10 Gy, 2hr)
0 60 120 0 60 120
minutes of 1 μM doxorubicin treatment
C
+ 1 μM doxorubicin
0 0 1 10 100 μM wortmannin
p53 pS15
p53
actin
1 2 3 4 5


A
BT (ATM+)
L3 (ATM-)
0 0.5 1 2 4 0 0.5 1 2 4 hr
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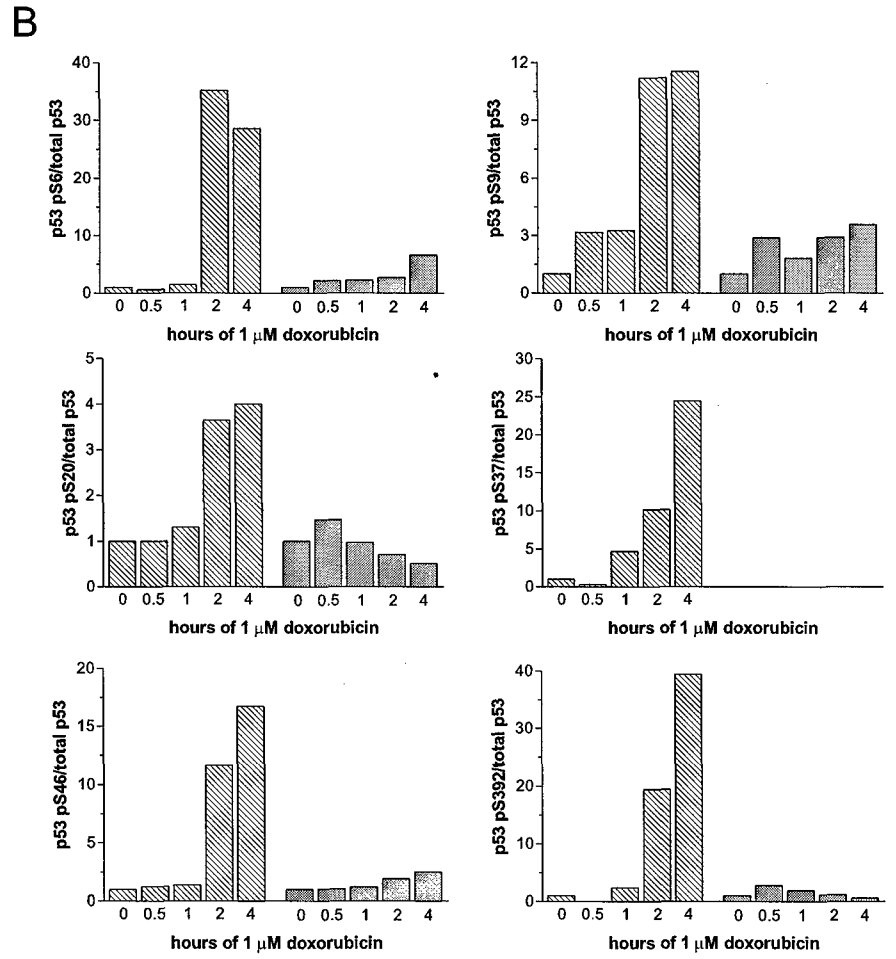
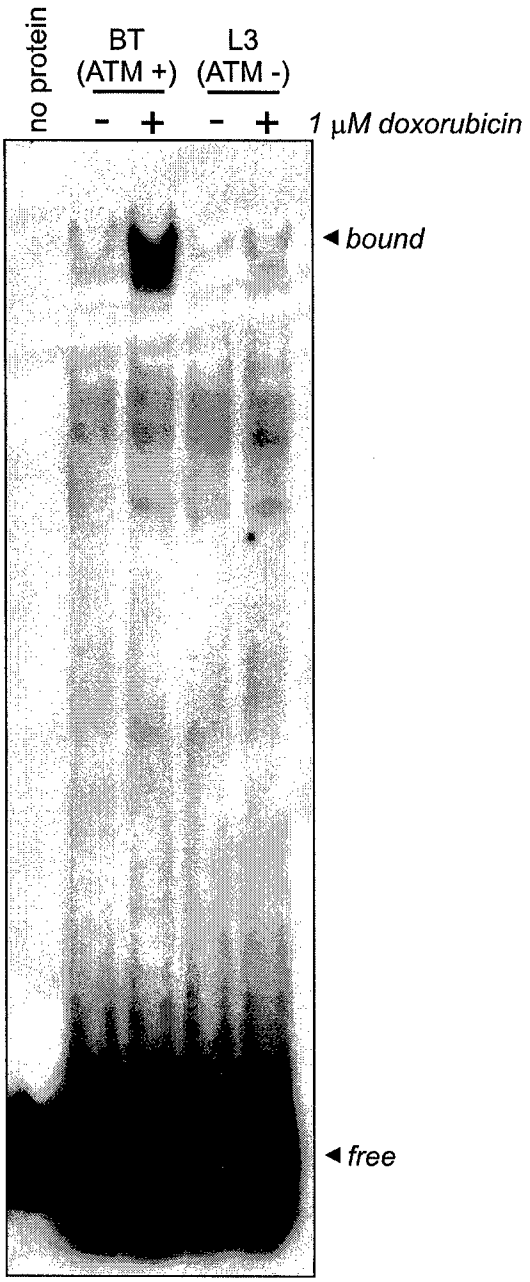
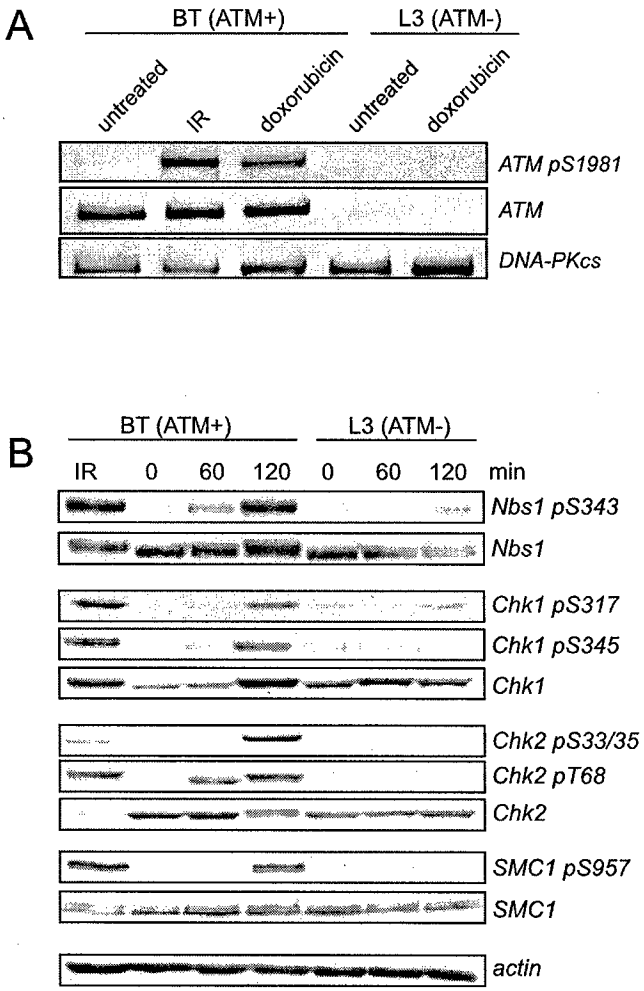
p53 pS9
p53 pS20
p53 pS37
p53 pS46
p53 pS392
total p53 (DO-1)


B
p53 pS6/total p53
p53 pS9/total p53
p53 pS20/total p53
p53 pS37/total p53
p53 pS46/total p53
p53 pS392/total p53
0 0.5 1 2 4 0 0.5 1 2 4
hours of 1 μM doxorubicin

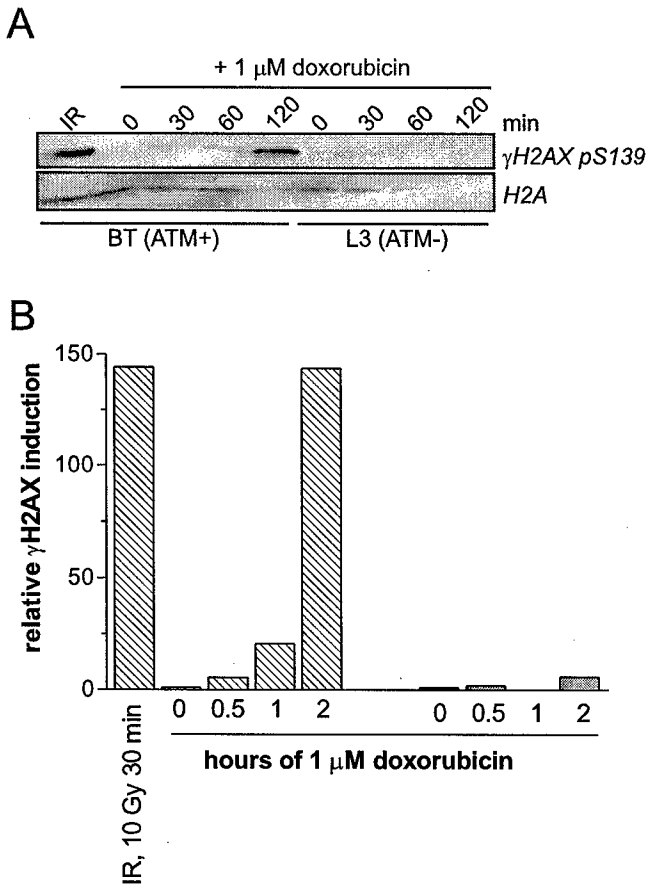
Kurz and Lees-Miller, Figure 3

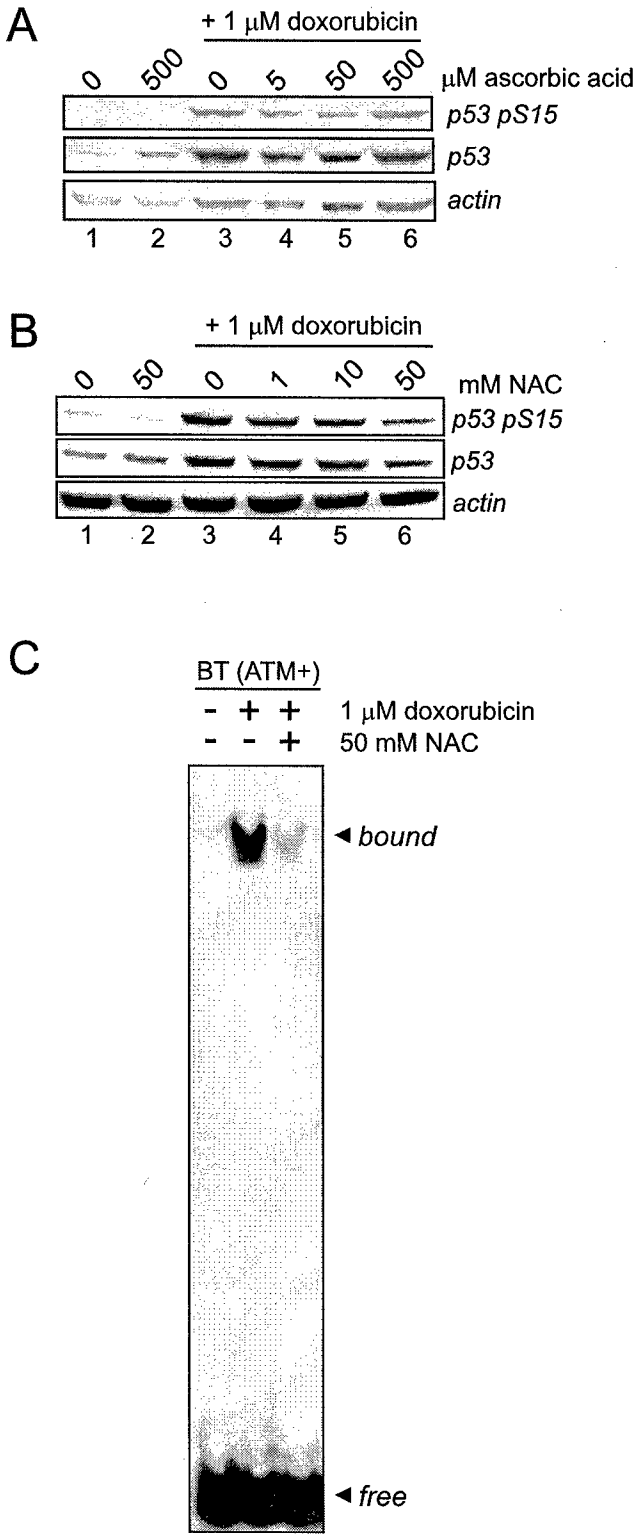


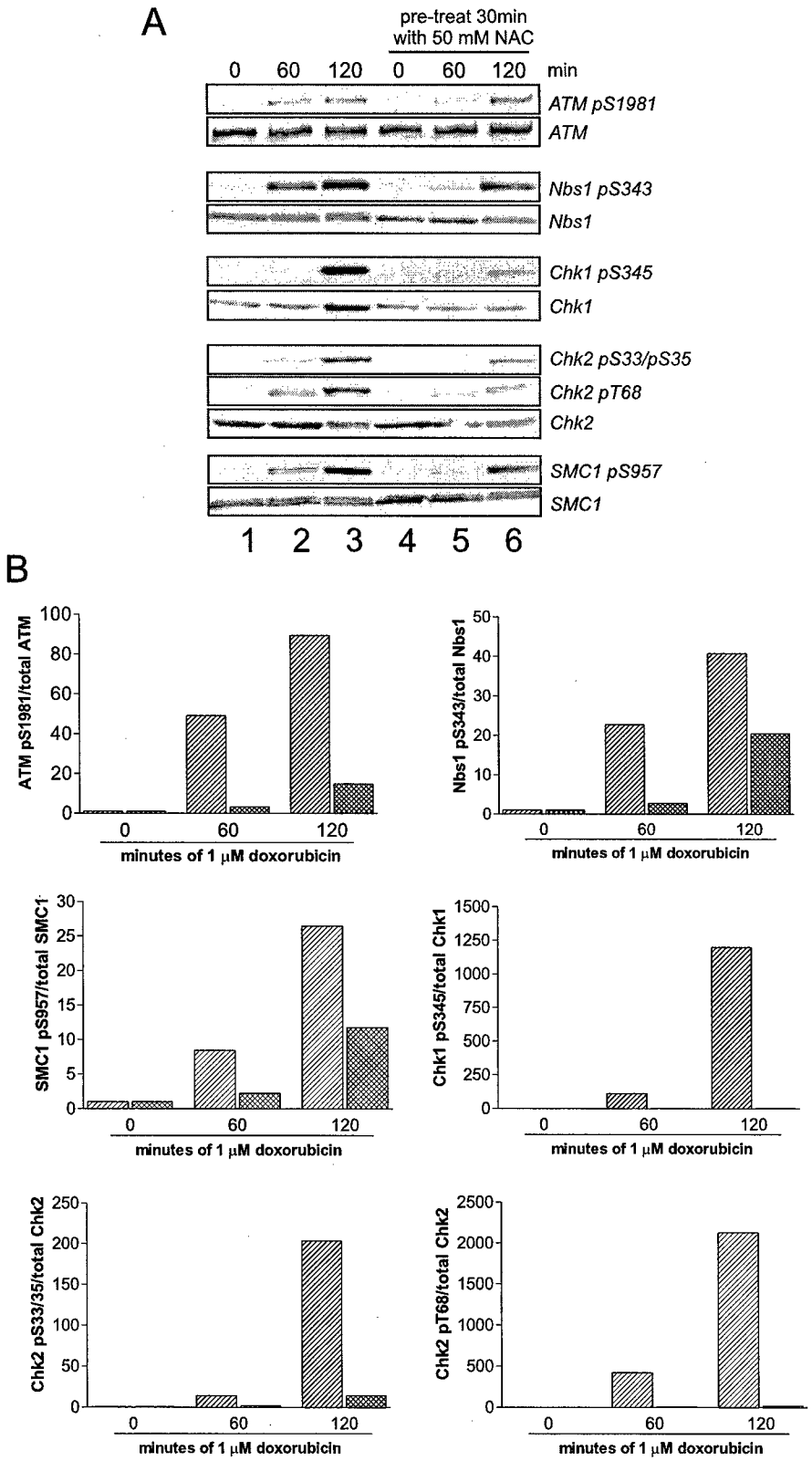
Kurz and Lees-Miller, Figure 4



Kurz and Lees-Miller, Figure 5




A
+ 1 μM doxorubicin
0
500
0
5
50
500
μM ascorbic acid
p53 pS15
p53
actin
1
2
3
4
5
6
B
+ 1 μM doxorubicin
0
50
0
1
10
50
mM NAC
p53 pS15
p53
actin
1
2
3
4
5
6
C
BT (ATM+)
- + +
- - +
1 μM doxorubicin
50 mM NAC
bound
free


A
pre-treat 30min
with 50 mM NAC
0 60 120 0 60 120 min
ATM pS1981
ATM
Nbs1 pS343
Nbs1
Chk1 pS345
Chk1
Chk2 pS33/pS35
Chk2 pT68
Chk2
SMC1 pS957
SMC1
1 2 3 4 5 6
B
ATM pS1981/total ATM
Nbs1 pS343/total Nbs1
SMC1 pS957/total SMC1
Chk1 pS345/total Chk1
Chk2 pS33/35/total Chk2
Chk2 pT68/total Chk2
minutes of 1 μM doxorubicin

Appendix 7: Reviewer comments (email communication) on **Kurz** et al manuscript submitted to Journal of Biological Chemistry (Appendix 6)

Subject:
From the JBC re: Manuscript M4:06879
From:
mmarkham@asbmb.faseb.org
Date:
Fri, 16 Jul 2004 07:30:28 -0500
To:
kurz@ucalgary.ca

M4:06879
Dear Dr. Kurz:

Your manuscript entitled "Doxorubicin activates ATM-dependent phosphorylation of multiple downstream targets in part through the generation of reactive oxygen species" has been reviewed by a member of the Editorial Board. I am pleased to report that the reviewer's overall evaluation was positive. The reviewer had several concerns, however, that must be addressed before a final decision can be made concerning your manuscript.

We welcome a revised manuscript that addresses the concerns of the reviewer. Please return your revised manuscript via the JBC Electronic Submission site (http://www.jbc.org/submit). The manuscript must be accompanied by a cover letter in which you provide a point-by-point discussion of the changes made. Please note that the revised manuscript must be submitted within four months or it will be considered a new manuscript.

You may submit the revised manuscript as a single PDF file or as individual source files for text and figures. However, source files will be required if the manuscript is accepted for publication. If you submit only a PDF file for the revised manuscript, there may be a delay in time to print publication due to the additional submission and processing of source file. Therefore you are encouraged to submit source files for the revised manuscript. The following points should be considered when preparing these source files:

*Your text files must be created in Microsoft Word version 6.0 or later.
*Graphic files must be in high resolution TIFF or EPS format.
*Complete instructions of preparation of text and graphic files can be found at
www.jbc.org/misc/itoa.shtml

It is crucial that you review the revised version of your manuscript with great care before submission. If your paper is accepted, we will publish your manuscript in JBC Papers in Press on the day of acceptance. A description of JBC Papers in Press can be found at http://www/jbc/org.

Thank you for giving us the opportunity to consider your work. I look forward to receiving your revised manuscript.

Peggy J. Farnham
Associate Editor

Comments for author:

This manuscript describes effect of Doxorubicin (DOX) on the activation of ATM and ATM-dependent signals using ATM-proficient and ATM-deficient human lymphoblastoid cells. The data show the following points.

1. Doxorubicin (DOX) treatment induced ATM-dependent phosphorylation at serine residues 6,9, and 20, and stabilization of p53.

2. DOX treatment induced phosphorylation of ATM at S 1981. DOX treatment induced ATM-dependent phosphorylation of Chk2 at Th 68, Nbs1at S 343 in a short period, then that of Chk1at S 317/345, Chk2 at S 33/35, and SMC1at S 957.

3. Phosphorylation of HistoneH2AX was also observed at S139 by DOX.

4. Pre-treatment with ascorbic acid had no apparent effect on the phosphorylation and stabilization of p53, pre-treatment with NAC as well as PDTC (hydroxyl radical scavengers) suppressed those of p53, and decreased or delayed the phosphorylation of ATM, Nbs1, Chk1, Chk2, and SMC1.

Experiments are well organized and the data obtained seems reliable. Almost of the data are based on previously published reports concerning RI-induced activation of ATM-dependent signals. Furthermore, recently ATM-dependent activation of NF-kB by DOX has reported (Mol. Cell. Biol., 2004). Production of ROS by DOX-treatment is well known. The original and interesting point in this manuscript is that hydroxyl radical may be involved in ATM-dependent signals in response to DOX-induced DNA damage.

Significance of ATM was shown clearly in Figs 1- 5. However, data concerning the effect of NAC on these signals are not sufficient to indicate the significance of ROS during DNA-repair. Firstly, it seems likely that ROS is partially involved in the ATM-dependent signal pathways. But, the reason for partial suppression of Nbs1 pS343 and SMC1 pS947 by NAC should be explained. Secondly, data should be shown if NAC suppresses DNA damage by DOX directly or suppresses the ATM-dependent signal pathways downstream of DNA-damage. Suppression of gamma-H2AX phosphorylation at S139 was observed by ATM (-) cells in response to DOX (Fig. 5), while, effect of NAC on the ATM-dependent phosphorylation of gamma-H2AX at S139 induced by DOX was not shown. It should be added.